

**United States Department of Agriculture
Food Safety and Inspection Service, Office of Public Health Science**

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Title: Screening and Determination of Multiple Hormones in Beef Muscle by LC/MS/MS		
Revision: 01	Replaces: CLG-HRM.00	Effective: 11/05/18

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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 C₁₈ SPE column, filtered, and injected onto LC/MS/MS (APCI).

2. Applicability

This method is suitable for the screening and determination of melengestrol acetate (MGA), megestrol (MEG), hexestrol (HEX), and zeranol (ZER) in beef muscle at levels listed in J.3.

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 numbers 14-959-49B 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.

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- 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. C₁₈ 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 – Models 1200 and 6410, Agilent Technologies
 - b. Liquid chromatography column - Poroshell 120 EC-C18, 100 x 3.0 mm, 2.7 µm, catalog number 695975-302, Agilent Technologies.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted. The maximum length of time that a working reagent shall be used is 1 year unless the laboratory has produced extension data

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, for sample evaporation.
2. Solutions
- a. Methanol/Water - 50/50 v/v
Mix 500 mL of methanol with 500 mL of DI water. Solution may be stored up to 1 year at room temperature.

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

Mix 400 mL of methanol with 600 mL of DI 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. In-house prepared standards shall be assigned an expiration date that is no later than the stability stated in the method. The maximum length of time that an in-house prepared standard shall be used is 1 year unless the laboratory has produced extension data.

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.

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

Calculate the volume of each of the concentrated individual stock solutions necessary to prepare 50 mL of a 1.00ng/μL solution (~500μL). Using an automated micropipettor, accurately measure these volumes into a single 50 mL volumetric flask. Bring to volume with methanol and mix well.

- c. 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.

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Table 1. Preparation of Fortification Standards

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

d. Working Standards for the Calibration Curve:

To prepare calibration standards, use automated micropipettors to pipette 100 μ L of the standard listed in the second column of Table 2 into 2 mL autosampler vials. Add 900 μ L of 40/60 (v/v) methanol/water to each autosampler vial and vortex. The final volume is 1000 μ L. After preparation, the calibration curve standards are stable for one year at $\leq -10^{\circ}$ C.

Note: Calibrations standards may be prepared in small quantities and stored for no longer than a 3 month period at 10° C.

Table 2. Preparation of Calibration Standards

Calibration Standard	From Table 1 Pipette 100 μ L of:	μ L of 40/60(v/v) MeOH/H ₂ O	Hormone ppb in 2g tissue	Final Conc in 1000 μ L (ng/mL)
STD#1	AHORM#1 (0.010 ng/ μ L)	900	0.500	1.0
STD#2	AHORM#2 (0.020 ng/ μ L)	900	1.00	2.0
STD#3	AHORM#3 (0.040 ng/ μ L)	900	2.00	4.0
STD#4	AHORM#4 (0.080 ng/ μ L)	900	4.00	8.0

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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 ($< -10^{\circ}\text{C}$) 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 ($< -10^{\circ}\text{C}$) prior to analysis.

F. ANALYTICAL PROCEDURE

1. Preparation of Controls and Samples

- a. Screening – Weigh one portion each for a blank (negative control), and a 0.5 ng/g recovery (positive control). See fortification table in Appendix J.5. for complete details for preparation of fortified tissues.
- b. Determination - Weigh one portion each for a blank (negative control), and a 2 ng/g recovery (positive control) See fortification table in Appendix J.5 for complete details for preparation of fortified tissues.

2. Extraction Procedure

- a. Weigh 2.0 ± 0.2 g of homogenized tissue into a 50 mL polypropylene centrifuge tube.
- 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 $4 \pm 1^{\circ}\text{C}$.
- 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 $4 \pm 1^{\circ}\text{C}$.
- 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 $\sim 1 - 2$ mL or until the sample volume remains unchanged using an evaporator set at 40°C .

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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 $4 \pm 1^\circ\text{C}$ 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 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.

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- d. Dry the column for ~1 min (longer if needed).
- e. Elute the analytes off the columns into glass or polypropylene tubes with 5 mL ACN at a rate of ~2 drops/second. Dry the column for ~2 min.
- f. Concentrate the eluate to dryness using an evaporator set at 40 °C.
- g. Reconstitute with 1000 µL of methanol/H₂O (40/60, v/v). Vortex for 15 seconds and sonicate for 2 min.
- h. Filter the solution with a BD syringe through an Acrodisc PTFE filter (13 mm, 0.2 µm) into a 2 mL autosampler vial.

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. LC Operating Parameters
 - i. Oven temperature: 40°C
 - ii. Mobile Phase: Isocratic Elution MeOH/H₂O/ACN (30/40/30)
 - iii. Flow: 0.400 mL/min
 - iv. Autosampler temperature: 10°C
 - v. Injection volume: 20 µL
 - vi. Run time: 12.5 min
- b. MS/MS Operating Parameters
 - i. Gas Temperature: 325°C
 - ii. Vaporizer: 350°C
 - iii. Gas Flow: 4 L/min
 - iv. 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.J.

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Table 3. 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 & Screening Ion

Table 4. 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 & Screening Ion

7. Sample Set

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

- a. Calibration standard(s).
- b. Negative Control/Blank sample.
- c. Positive Control/Recovery sample
- d. Check sample (if necessary)
- e. Samples.
- f. Calibration standard at the appropriate level.

G. CALCULATIONS / IDENTIFICATION

1. Screening

- a. The screening ion for each analyte must be present. The required ion for each compound is listed in bold in Tables 3 and 4.

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- b. The retention times for the screening ions in the recoveries must match the retention time of the screening ions in the standard within 5%. Retention time for the screening ions in the samples must match the retention time of the screening ions in the recovery or standard within 5%.
- c. The screening ions must have a signal-to-noise ratio ≥ 3 . This may be verified by visual inspection.
- d. A sample is screened positive for an analyte if the sample response equals or exceeds the recovery level.

2. Determination

- a. The quantitation ion for each analyte must be present. The required ion for each compound is listed in bold in Tables 3 and 4.
- b. The retention times for the quantitation ions in the recoveries must match the retention time of the quantitation ions in the standard within 5%. Retention time for the quantitation ions in the samples must match the average retention time of the quantitation ions in the standards within 5%.
- c. The quantitation ions must have a signal-to-noise ratio ≥ 3 . This may be verified by visual inspection.
- d. Construct calibration curves by plotting the quantitation ion peak responses against the analyte concentrations.
- e. 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:

$$Y = \text{peak response} \quad X = \text{concentration of sample (ng/g)}$$

The coefficient of correlation (r^2) must be ≥ 0.990 .

- f. The baseline as determined by automated data system analysis should be individually inspected for each standard, control and sample.
- g. The final sample concentration (except for control and fortified samples) is corrected for mass by taking into account the mass difference of the individual samples to the nominal 2 g samples used to calculate the concentrations of analyte in the calibration standards.

$$\text{Concentration in tissue (ng/g)} = \frac{\text{Experimental ng/g} \times 2}{\text{Weight of sample (g)}}$$

- h. Determination criteria are required only for analytes that are to be quantitated in the sample set.

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H. SAFETY INFORMATION AND PRECAUTIONS

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

<i>Procedure Step</i>	<i>Hazard</i>	<i>Recommended Safe Procedures</i>
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. Screening Criteria
 - i. For sample reporting purposes, the analytes of interest in the recovery must meet the screening criteria in Section G.

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- ii. The blank must be negative using the criteria in Section G.
- iii. The fortified recovery of the analyte must exceed 10% of the standard.
- iv. The level of the screening ions in the blank must be less than 10% of the level of the standard.

b. Determination Criteria

- i. Calibration curves should have a Coefficient of Determination (r^2) ≥ 0.990 .
- ii. The recovery for fortified samples for Determination should be 60-110% with coefficients of variation (CV) $\leq 20\%$.
- iii. For the blank, verify the absence of analytes above 10% of the recovery concentration.

2. Critical Control Points and Specifications

<u>Record</u>	<u>Acceptable Control</u>
a. Sample weight	2.0 \pm 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

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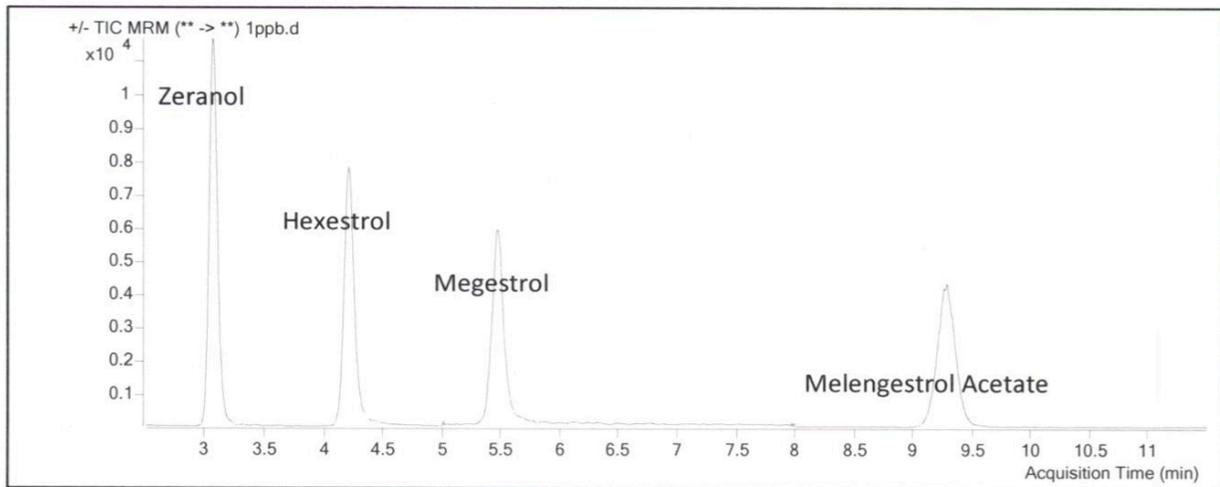
J. APPENDIX

1. References

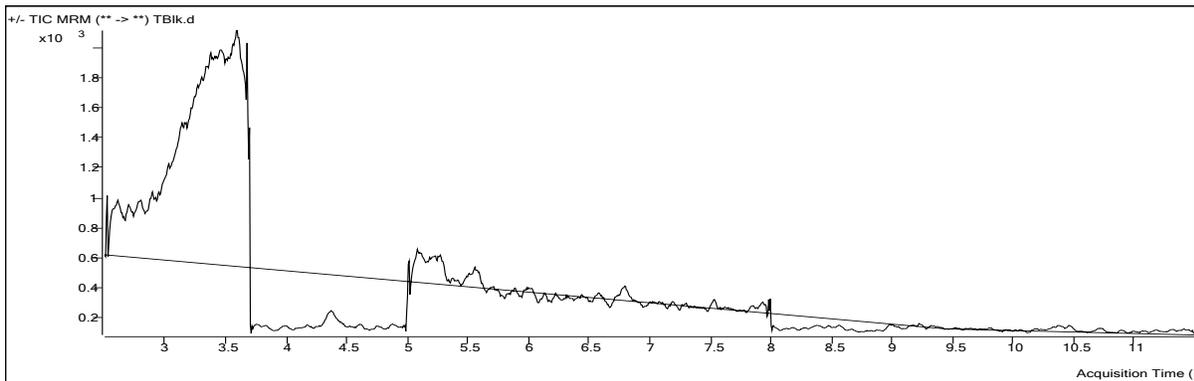
Chu, Pak-Sin and Tricia Johnson. LC-MS/MS Determination of Multiple Hormones in Beef Muscle. CVM Office of Research, Division of Residue Chemistry, FDA. November 2012.

2. Chromatograms/spectra

a. 1 ppb std



b. Blank sample



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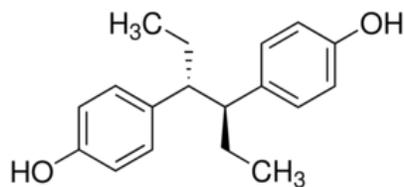
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3. Minimum Level of Applicability for all analytes:

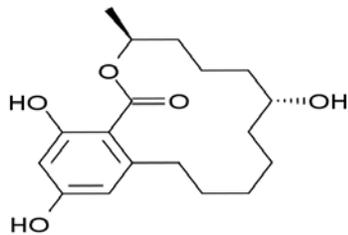
- a. Screen: 0.5 ng/g
- b. Determination: 1 ng/g

4. Structures of Hormones

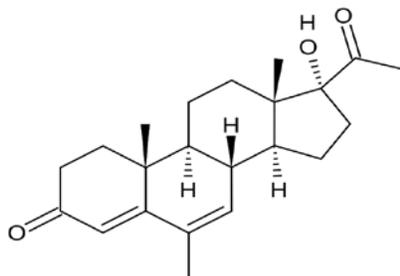
a. Hexestrol



b. Zeranol



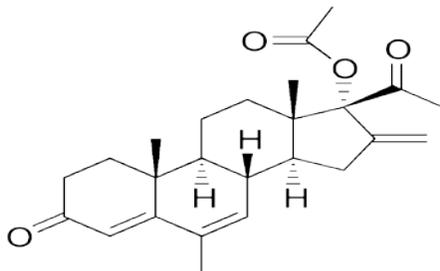
c. Megestrol



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d. Melengestrol Acetate



5. Fortification of Tissue Table

Sample Fortification Concentration ng/g	AHORMS Spiking Standards			
	AHORMS #1 (0.01ng/μL)	AHORMS #2 (0.02 ng/μL)	AHORMS #3 (0.04 ng/μL)	AHORMS #4 (0.08 ng/μL)
0.5	100 μl	50 μl	25 μl	
1.0	200 μl	100 μl	50 μl	25 μl
2.0		200 μl	100 μl	50 μl
3.0			150 μl	75 μl
4.0			200 μl	100 μl

K. APPROVALS AND AUTHORITIES

1. Approvals on file.
2. Issuing Authority: Director, Laboratory Quality Assurance Staff.