

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

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Revision: 00	Replaces: NA	Effective: 01/03/05

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**A. INTRODUCTION**

1. Theory

Tissues are homogenized in sodium acetate buffer, digested with  $\beta$ -glucuronidase, mixed with acetonitrile, and then centrifuged. A three-phase extraction mixture is obtained after addition of methylene chloride and hexane in which trenbolone metabolites are extracted into the middle acetonitrile layer. This extract is further purified using both C18 and silica gel SPE columns. The purified extract is derivatized by co-injection with a trimethylsilyl (TMS) derivatizing agent into a GC/ion-trap MS and analyzed by full scanning product ion MS.

2. Applicability

This method is applicable to  $\alpha$ -trenbolone in bovine liver and  $\beta$ -trenbolone in bovine muscle at  $\geq 5$  ppb.

**B. EQUIPMENT**

Equivalent apparatus and instrumentation may be substituted for those listed below:

1. Apparatus

- a. Centrifuge - IEC-Centra-GP8R.
- b. pH meter - #215 equipped with a Accumet micro combination electrode, No.13-620-95, Denver Instrument Co.
- c. Vortex mixer- Genie 2, Fisher Scientific.
- d. Nitrogen evaporator - TurboVap LV, Zymark.
- e. Balance - #MT5, Mettler.
- f. Ultrasonic bath - Aquasonic #150T, VWR.
- g. Mechanical shaker - Eberbach two-speed, flat bed, VWR.
- h. Incubator - #100, Labline.
- i. SPE column - Silica gel, Bond Elute, 500 mg, 10 mL, #1211-3036, Varian.
- j. SPE column - C18, Bond Elute, 500 mg, 6 mL, #1210-2052, Varian.
- k. Repeater pipette - # 4780, Eppendorf.
- l. Dispenser tips - 12.5 mL, #13-683-61E, Fisher Scientific.
- m. Dispenser tips - Finntip, 200  $\mu$ L extended #9400100, Thermo Labsystems.
- n. SPE vacuum manifold - 12 - 24 place, Supelco.
- o. Homogenizer - Ultra turrax T-18, IKA Works.
- p. Graduated cylinder - 50 mL, class B.
- q. Volumetric flask - Class A, 25 mL.

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- r. Repipettors - to cover the range 20  $\mu$ L to 5000  $\mu$ L.
  - s. Centrifuge tubes - 15 mL glass, #73790, Kimble, for Zymark, TurboVap.
  - t. Centrifuge tubes - 50 mL polypropylene, Falcon.
  - u. Blender - Waring Products Division, New Hartford, CT.
  - v. Robot Coupe<sup>®</sup> processor - Robot Coupe U.S.A., Inc., Jackson, MS.
  - w. Syringe - 10  $\mu$ L, 26 ga., 70 mm needle, P/N-002003-10F, SGE.
2. Instrumentation
- a. Ion-trap mass spectrometer - GCQ-Polaris, Thermo-Finnigan.
  - b. GC column - DB-5 ms, 30m, 0.25 mm i.d., 0.25  $\mu$ m film thickness, J&W Scientific.

**C. REAGENTS AND SOLUTIONS**

Equivalent reagents and solutions may be substituted for the following unless otherwise indicated.

1. Reagents
- a.  $\beta$ -Glucuronidase - Type H-2 from Helix pomatia, Sigma.
  - b. Sodium acetate (anhydrous) - reagent grade, Fisher Scientific.
  - c. Acetonitrile - GC grade, Mallinkrodt.
  - d. Hexane - GC grade, Mallinkrodt.
  - e. Dichloromethane - GC grade, Mallinkrodt.
  - f. Methanol - GC grade, Mallinkrodt.
  - g. Acetone - GC grade, Mallinkrodt.
  - h. Toluene - GC grade, Mallinkrodt.
  - i. Ethyl alcohol - GC grade, Mallinkrodt.
  - j. Sodium hydroxide - reagent grade, J.T. Baker.
  - k. Water - 18 mega-ohm, Purelab Plus, U.S. Filter.
  - l. Glacial acetic acid - reagent grade.
  - m. BSTFA [N,O-bis(trimethylsilyl)-trifluoroacetamide] - #38830, Pierce.
  - n. TMSI (N-(trimethylsilyl) imidazole) - #18050, Alltech.
  - o. Ethyl Acetate - GC Grade, Mallinckrodt.
2. Solutions
- a. 0.04 M Sodium acetate buffer:  
Dissolve 3.28 g sodium acetate (anhydrous) in 1 L of water.

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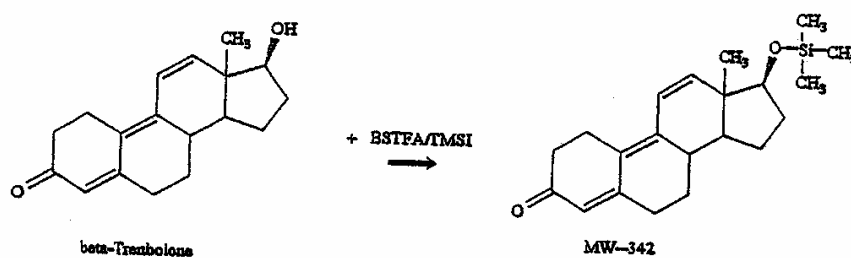
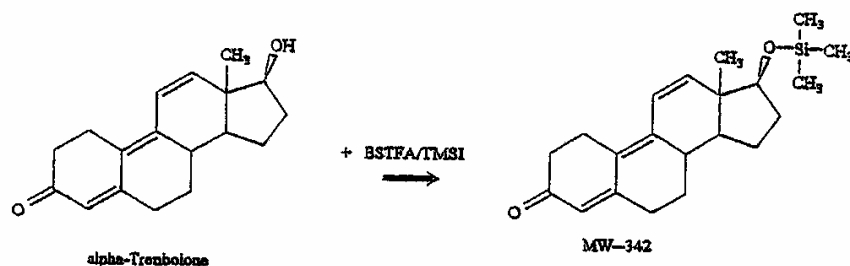
- b. 6 M Sodium hydroxide:  
Dissolve 60 g of sodium hydroxide in 250 mL in water.
- c. Methanol:water (40:60):  
Combine 40 parts methanol with 60 parts water.
- d. Methanol:water (80:20):  
Combine 80 parts methanol with 20 parts water.
- e. Acetone:toluene (10:90):  
Combine 10 parts acetone with 90 parts toluene. Make fresh daily.
- f. Acetone:toluene (20:80):  
Combine 20 parts acetone with 80 parts toluene. Make fresh daily.
- g. BSTFA/TMSI (5%):  
Add 50  $\mu$ L TMSI to 1 mL of BSTFA. Store under N<sub>2</sub> between 2 - 8 °C.

**D. STANDARDS**

- 1. Source
  - a.  $\alpha$ -Trenbolone (17- $\alpha$ -hydroxyestra-4,9,11-triene-3-one)  
Community Reference Laboratory, Bilthoven, Netherlands.
  - b.  $\beta$ -Trenbolone (17- $\beta$ -hydroxyestra-4,9,11-triene-3-one)  
Community Reference Laboratory, Bilthoven, Netherlands or  
Sigma-Aldrich # T3925.
  
- 2. Structure

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3. Preparation

Note: Equivalent standards and solutions may be substituted for the following. Follow the manufacturer's instructions for reconstituting in glass ampoules if different from the instruction given below.

a. 17 -  $\alpha$  Trenbolone Standard Solutions

i. 17 -  $\alpha$  Trenbolone Stock Solutions (100  $\mu$ g/mL):

Add 1.00 mL of ethyl alcohol to an ampoule containing 0.1 mg. of 17- $\alpha$  Trenbolone. Vortex the ampoule for at least 1 min. Place the ampoule in an ultrasonic waterbath for at least 5 min. Stable for 6

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months, if stored in freezer.

- ii. 17 - alpha Trenbolone fortification Solution (0.25  $\mu\text{g}/\text{mL}$ ):

Transfer 62.5  $\mu\text{L}$  of stock solution in to a 25 mL volumetric flask and dilute to volume with methanol. Stable for 6 mos.

- b. 17 - beta Trenbolone Standard Solutions

- i. 17 - beta Trenbolone Stock Solution (100  $\mu\text{g}/\text{mL}$ ):

Add 1.00 mL of ethyl alcohol to an ampoule containing 0.1 mg of 17-beta Trenbolone. Vortex the ampoule for at least 1 min. Place the ampoule in an ultrasonic waterbath for at least 5 min. Stable for 6 months if stored in freezer.

- ii. 17 - beta Trenbolone fortification solution (0.25  $\mu\text{g}/\text{mL}$ ):

Transfer 62.5  $\mu\text{L}$  of stock solution in to a 25 mL volumetric flask and dilute to volume with methanol. Stable for 6 mos.

- c. 17-alpha and 17- beta Trenbolone External standards (5 ppb):

Transfer 100  $\mu\text{L}$  each of the 17-alpha and 17-beta fortification solutions separately to a 15 mL centrifuge tube and dry. Add 100  $\mu\text{L}$  ethyl acetate. Solution is stable for one week.

## **E. SAMPLE PREPARATION**

1. Homogenize liver samples in a Waring blender.
2. Homogenize muscle samples in a Robot Coupe<sup>®</sup>.

## **F. ANALYTICAL PROCEDURE**

1. Sample Cleanup

- a. Weigh 5.0 g homogenized or ground tissue into a 50 mL polypropylene centrifuge tube.

Note: Prepare controls (blank and 5 ppb recovery) by weighing out two blank tissues. Fortify one blank liver with 100  $\mu\text{L}$  of fortification solution of 17 - alpha Trenbolone 0.25  $\mu\text{g}/\text{mL}$  (D.3.a.ii) or if muscle is used fortify one blank muscle with 17 - beta Trenbolone 0.25  $\mu\text{g}/\text{mL}$  (D.3.b.ii).

- b. Let stand at room temperature for 15 minutes.
- c. Add 11 mL of 0.04 M sodium acetate buffer.
- d. Homogenize until smooth (20 - 40 sec.). Rinse probe with approximately 1 mL of 0.04 M sodium acetate buffer and add directly to the sample tube.
- e. Adjust pH of tissue/buffer mixture to 4.3 - 4.8 by adding approximately 70  $\mu\text{L}$  of glacial acetic acid.
- f. Add 20  $\mu\text{L}$  of  $\beta$ -glucuronidase solution to each sample. Vortex approximately 10

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- sec. Let stand at room temperature for 30 min.
- g. Incubate overnight at  $37 \pm 1$  °C.
  - h. After incubation, add 20 mL acetonitrile to each tube. Place on mechanical shaker and shake on high for 5 min.
  - i. Centrifuge at approximately 2000g for 10 min. at 18 °C.
  - j. Decant the supernatant into a 50 mL polypropylene tube.
  - k. For liver samples, add 1.00 mL of 6 M sodium hydroxide. For muscle samples add 0.50 mL of 6 M sodium hydroxide. Shake by hand for approx. 5 sec.
  - l. Add 8 mL of hexane and 2 mL of dichloromethane to the supernatant.
  - m. Shake on high for one min. and centrifuge at approximately 2000g for 5 min. at 18 °C.
  - n. Transfer the middle layer to a clean 50 mL polypropylene centrifuge tube.
  - o. Add a further 5 mL of acetonitrile to remaining hexane/ dichloromethane/ aqueous layers.
  - p. Shake on high speed for one min. and centrifuge for approximately 2000g for 5 min. at 18 °C.
  - q. Add middle layer to previous acetonitrile layer.
  - r. Add 5 mL water to the combined acetonitrile layers.
  - s. Shake on high for one min. and centrifuge for approximately 2000g for 5 min. at 18 °C.
  - t. Discard bottom aqueous layer.
  - u. Evaporate extract to complete dryness at  $60 \pm 3$  °C under nitrogen.
  - v. Add 3 mL water to residue and let stand approximately 10 min. Continue dissolution by placing in ultrasonic bath for 5 min. Do not vortex.
  - w. Load C18 cartridges on vacuum manifold. Precondition cartridges with approximately 6 mL of methanol, followed by approximately 6 mL of water.
  - x. Load samples to cartridges. Rinse sample tube with 2 mL of water and add rinse to cartridge. Swirl, do not vortex.
  - y. Draw sample through the cartridge at approximately 1 - 2 drops per sec. Turn off vacuum when liquid level drops below top of bed.
  - z. Add 2 mL of 40:60 methanol:water to cartridge. Apply vacuum and allow solution to drain at 1 - 2 drops per sec. until completely through cartridge. Discard eluate.
  - aa. Place collection tubes under cartridges. Add 3 mL of 80:20 methanol:water to each cartridge. Apply vacuum and collect solution again at a rate of 1 - 2 drops per sec. Draw air through cartridge to ensure all the eluate is collected.
  - bb. Concentrate the eluate to approximately 100  $\mu$ L at  $60 \pm 3$  °C. Rinse the walls of the tube with methanol during the concentration. Do not dry.

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- cc. Dissolve the residue in 2 mL of toluene. Sonicate for 5 min. and then vortex for a minimum of 20 sec. to ensure the residue at the tube bottom is churned up.
- dd. Centrifuge at approximately 2000g for 10 min. at 18 °C.  
Note: Sample extracts may be stored overnight at < -10° C
- ee. Load silica cartridges on the vacuum manifold and pre-condition with approximately 10 mL of toluene.
- ff. Load sample on cartridge.
- gg. Draw sample through the cartridge at approximately 1 - 2 drops per sec. Turn off vacuum when liquid level drops below top of bed.
- hh. Add 4 mL of 10:90 acetone:toluene to cartridge. Draw solution through cartridge at a rate of approximately 1- 2 drops per sec. Turn off vacuum when liquid level drops below top of bed. Discard eluate.
- ii. Put eluate collection tubes under cartridges. Add 5 mL of 20:80 acetone:toluene to each cartridge. Maintain a flow of approximately 1-2 drops per sec. Draw air through cartridge to ensure all of the solution is drawn out of cartridge.
- jj. Evaporate eluate to dryness at  $60 \pm 3$  °C, rinsing the tubes with toluene during the concentration.
- kk. Dissolve the residue in 100  $\mu$ L of ethyl acetate.

2. Instrument Operating Parameters

Note: Typical operating parameters are given below. Instrumental parameters yielding equivalent analytical results may also be used.

a. Instrument Operating Parameters - Gas Chromatograph

- i. Inlet Temperature: 260 °C
- ii. Oven program: Initial column temp - 100 °C. Hold 1 min.  
Program to 280 °C at 20 °/min. Hold 15 min.
- iii. Purge-off time 1 min.

b. Instrument Operating Parameters - Mass Spectrometer

- i. Transfer line: 280 °C
- ii. Start time 12 min.
- iii. Micro scans 2
- iv. Max ion time 200
- v. Scan width 1.5d



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- vi. Collision energy 1.20
- vii. q 0.225
- viii. Precursor ion 342.1d
- ix. Product ion scan range 150 - 260d

3. Instrumental analysis of samples, controls and standards.

Note: Standards, controls, and samples are injected into the GC/MS/MS using the following procedure.

Draw a portion of the 100  $\mu$ L ethyl acetate solution to the 1  $\mu$ L mark on the needle barrel (B.1.w.). Pull up 0.2  $\mu$ L of air. Place the needle in the BSTFA/TMSI derivatization agent. Draw derivatization solution to the 3.4  $\mu$ L mark on the needle barrel. Place needle into injector port and inject.

- a. Perform a leak check.
- b. Inject a 5 ppb mixed external standard under MS/MS conditions. Verify the TIC S/N for  $\beta$ -trenbolone is  $\geq 10$ .
- c. If above conditions are not met re-inject external standard under scanning Q1 from 330 - 350d to obtain exact precursor mass and repeat b. above.
- d. If S/N is still low perform an autotune.
- e. Product ion spectra should be easily recognizable.
- f. Inject the recovery and verify retention time and product ion spectra.
- g. Inject a solvent blank to ensure no carryover, then the tissue blank and sample extracts.

4. Chromatograms

See Section K. for chromatograms.

**G. CONFIRMATION**

1. Confirmation of  $\alpha$ - or  $\beta$ -trenbolone in a sample extract requires that the following criteria be met:

- a. Retention time of the extract peak must match that of the external standard within  $\pm 0.5\%$ .
- b. The following product ions must be present: 178, 193, 211, 226, and 237.
- c. The S/N ratio of the RIC is  $\geq 3$ .
- d. The spectra from the extract must visually match spectra from the external standards in the same data set.

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- i. There should be general correspondence between relative abundances or ranked abundances.
    - ii. There should be a general absence of non-specific ions in the sample spectrum.
  - e. The quality assurance positive and negative control samples must confirm and fail to confirm, respectively, for the presence of trenbolone.
2. Criteria for repeating an analysis
- Sample analyses may be repeated under the following conditions:
- a. The conditions described in G.1.d. are not met.
  - b. There is suspected carryover from a previous high concentration sample or standard. In this case, the sample should be re-analyzed after the cause of the carryover has been identified and measures taken to prevent its recurrence.
  - c. There is strong evidence of trenbolone presence, but extraneous ions with relative abundances exceeding those of one or more of the qualifying ions prevent a positive identification.

**H. SAFETY INFORMATION AND PRECAUTIONS**

- 1. Required Protective Equipment - Personal protective equipment (PPE) includes gloves, safety glasses and lab coat where applicable.
- 2. Hazards

<i>Procedure Step</i>	<i>Hazard</i>	<i>Recommended Safe Procedures</i>
Trenbolone	Undetermined	Wear PPE when handling standards.
Methanol Hexane Acetone Toluene Acetonitrile	Highly flammable. May produce toxic effects to skin, eyes and respiratory system.	Use under a fume hood away from all electric devices and open flames. Avoid breathing vapors.
Sodium Hydroxide Glacial Acetic Acid BSTFA/TMSI	Corrosive liquid	Prepare solutions in a fume hood. Wear PPE, and avoid contact with skin.
Dichloromethane	Possible carcinogen	Wear PPE, use in fume hood.

- 3. Disposal Procedures

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<i>Procedure Step</i>	<i>Hazard</i>	<i>Recommended Safe Procedures</i>
Methanol Hexane Acetone Toluene Acetonitrile	See above.	Collect waste in a sealed container and store in a cool well ventilated, flammable liquid storage area/cabinet for disposal in accordance to local, state and federal regulations.
Dichloromethane	See above.	Collect as above in a sealed container for chlorinated solvents.
Acetic acid/ Sodium Hydroxide	See above.	Collect waste in separate sealed containers and store in a cool, well ventilated, acid and base liquid storage areas/cabinets for disposal in accordance with local, state, and Federal regulations.

**I. QUALITY ASSURANCE PLAN**

1. Performance Standard

Refer to Section G. 1. for confirmation criteria.

2. Critical Control Points and Specifications

RESERVED

3. Readiness To Perform (FSIS Training Plan)

a. Familiarization

i. Phase I: Standards - Analyze external standard and blank solutions in duplicate on at least three different days and verify instrument response is adequate for confirmatory purposes:

(a) Reagent Blank

(b) 5 ppb external standard (0.25 ng/ $\mu$ L solution of D.3.c)

ii. Phase II: Fortified samples - Analyze on 3 different days:

(a) one blank liver,

(b) one 5 ppb fortified liver,

(c) one blank muscle, and

(d) one 5 ppb fortified muscle.

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Note: Phase I and Phase II may be performed concurrently.

- iii. Phase III: Check samples for analyst accreditation
  - (a) 6 check samples fortified at levels between 1-2 times Minimum Proficiency Level (MPL) using analytes and concentrations unknown to the analyst. These six unknowns shall be composed of three bovine liver and three bovine muscle tissues. Each set must include a positive control and a negative control.
  - (b) Approval from the Supervisor and the Laboratory Quality Assurance Manager (QAM) is required to commence official analysis.

b. Acceptability criteria.

Refer to I.1.

4. Intralaboratory Check Samples

a. System, minimum contents.

- i. Frequency: One per week per analyst when samples analyzed.
- ii. Records are to be maintained for review.

b. Acceptability criteria.

Refer to I.1.

If unacceptable values are obtained, then:

- i. Stop all official analyses by that analyst.
- ii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrices: Bovine liver and muscle.

b. Sample receipt, minimum weight: approximately 50 grams.

c. Condition upon receipt: chilled or frozen.

d. Sample storage:

- i. Time: 2 weeks for blended/homogenized samples.
- ii. Condition: frozen (less than -10 °C).

6. Sample Set

Each sample set must include the following:

- a. Negative liver/muscle control,
- b. Positive liver/muscle control,
- c. Samples.

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- 7. Sensitivity
  - a. Minimum proficiency level (MPL): 5 ppb.

**J. WORKSHEET**

An example of a worksheet can be found on the following page.

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**TRENBOLONE CONFIRMATORY WORKSHEET**

Analyst: _____	Trenbolone Fortified Std: _____
Date Started: _____	0.04 M Sod. Acetate: _____
Date Completed: _____	Glacial Acetic Acid: _____
Set Number: _____	B-Glucuronidase: _____
Species: _____	Acetonitrile: _____
Reviewed By (init and date): _____	6 M Sod. Hydroxide: _____
Sample Frz- _____ To Hold Frz- _____	Hexane: _____
Holding Frz- _____ To Frz- _____	Dichloromethane: _____
Turbo-Vap Temp. °C _____	Water: _____
	Methanol: _____
	40/60 Methanol/Water: _____
	80/20 Methanol/Water: _____
	Toluene: _____
	10/90 Acetone/Toluene: _____
	20/80 Acetone/Toluene: _____
	Ethyl Acetate: _____
	C18 Cartridge: _____
	Si Cartridge: _____
	Turbo-Vap: _____
	Micropipettor(s): _____
	Balance: _____
	Centrifuge(s): _____
	pH Meter: _____
	GC/MS: _____
BSTFA/TMSI 5% _____	Method File Name: _____
Trenbolone Ext. Std. _____	Injection Volume: _____

Sample Analysis Data

Sample No.	Lab. No.	Form No.	Tissue Type	Sample Wt. 5.00 Grams within 0.1gr.	Ret. Time	Ret. Time within 0.5% of Fort. Std	RIC > 3x Noise Level	Qualifying/ Base ion(s) Present?	Visual Match (w/ Ext. Std.)
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									

Note: Place a check mark where applicable to indicate a positive response. A check mark in each of the four columns represents a confirmation.

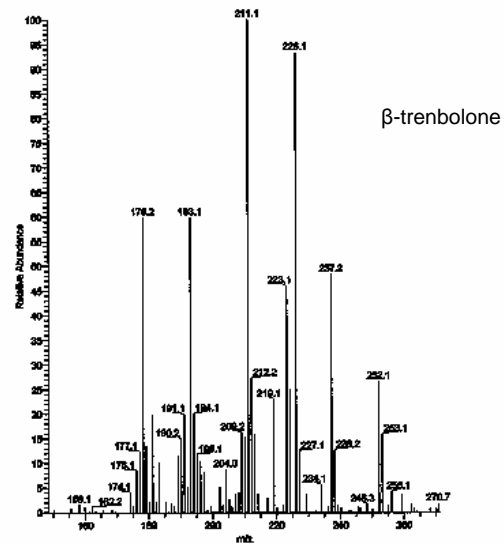
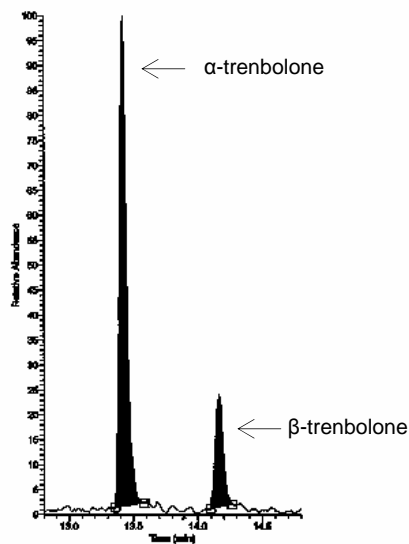
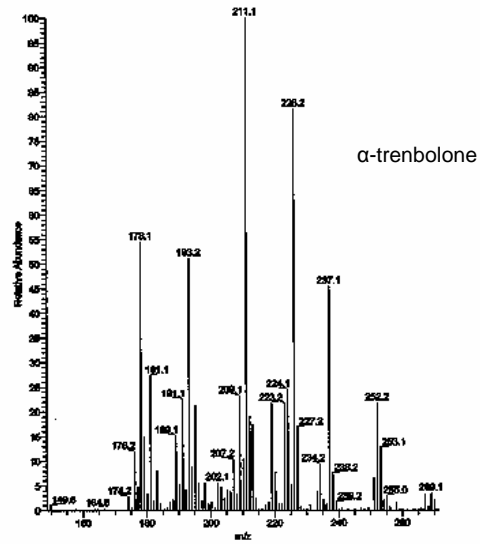
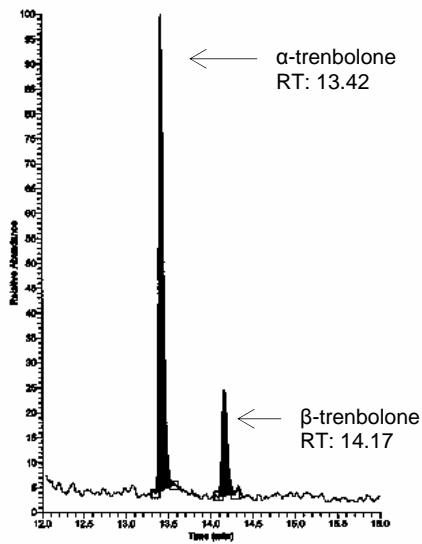
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K. APPENDIX

1. Chromatograms and Spectra

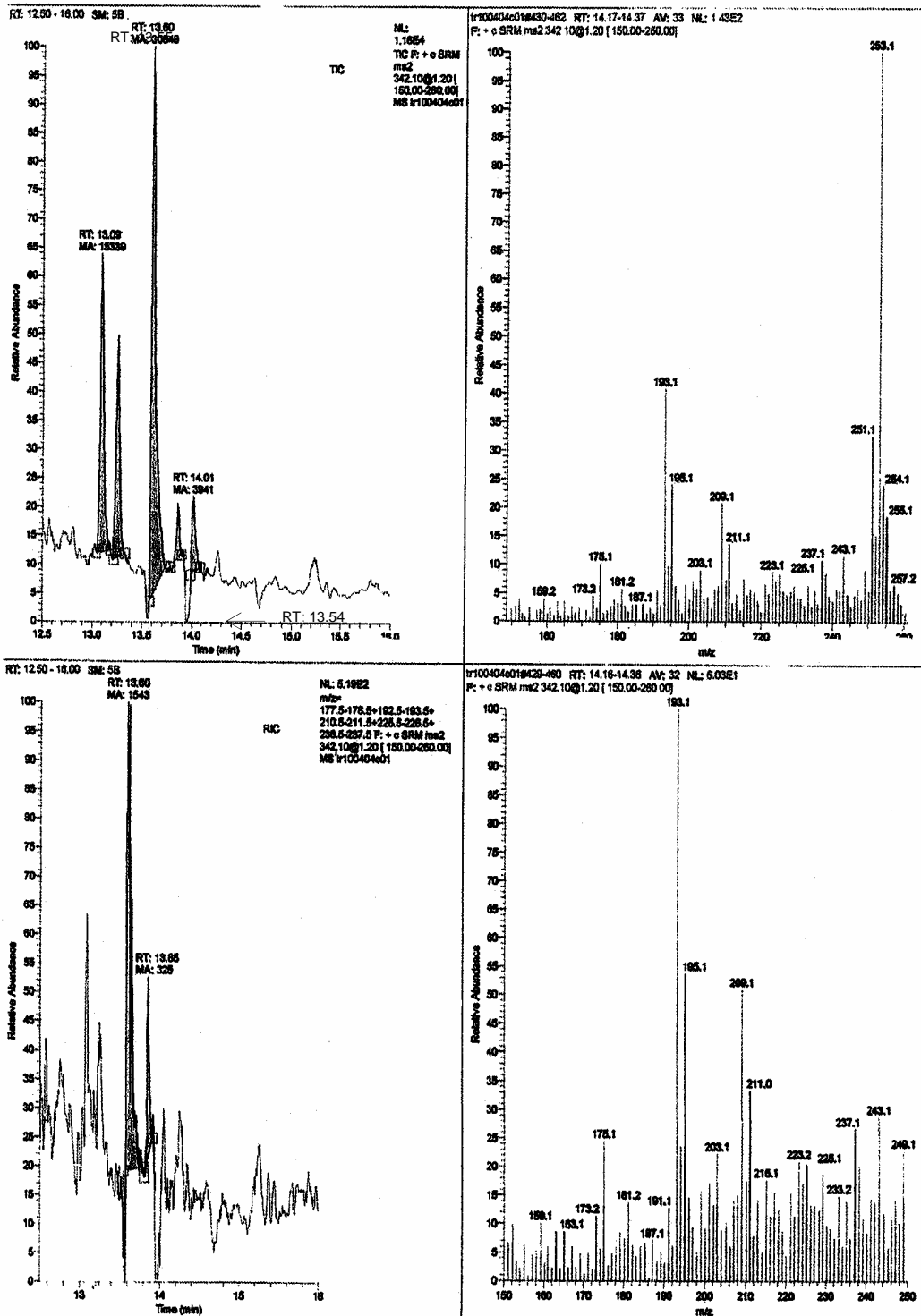
a. 5 ppb  $\alpha$ -trenbolone and  $\beta$ -trenbolone External Standard.



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b. Total Ion Chromatogram (TIC) of Blank Beef Liver (top trace) and Reconstructed Ion Chromatogram (RIC) - bottom trace.

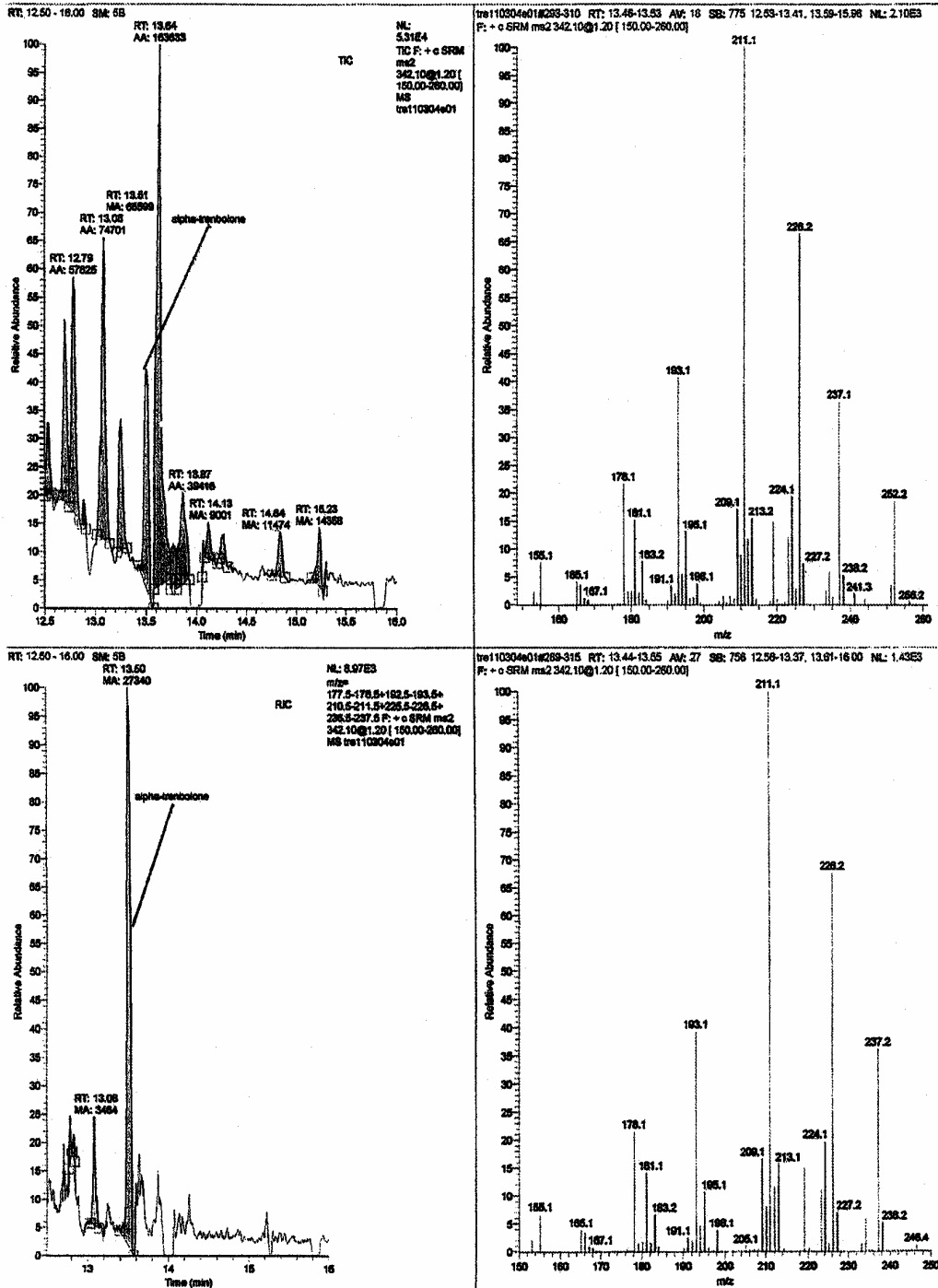




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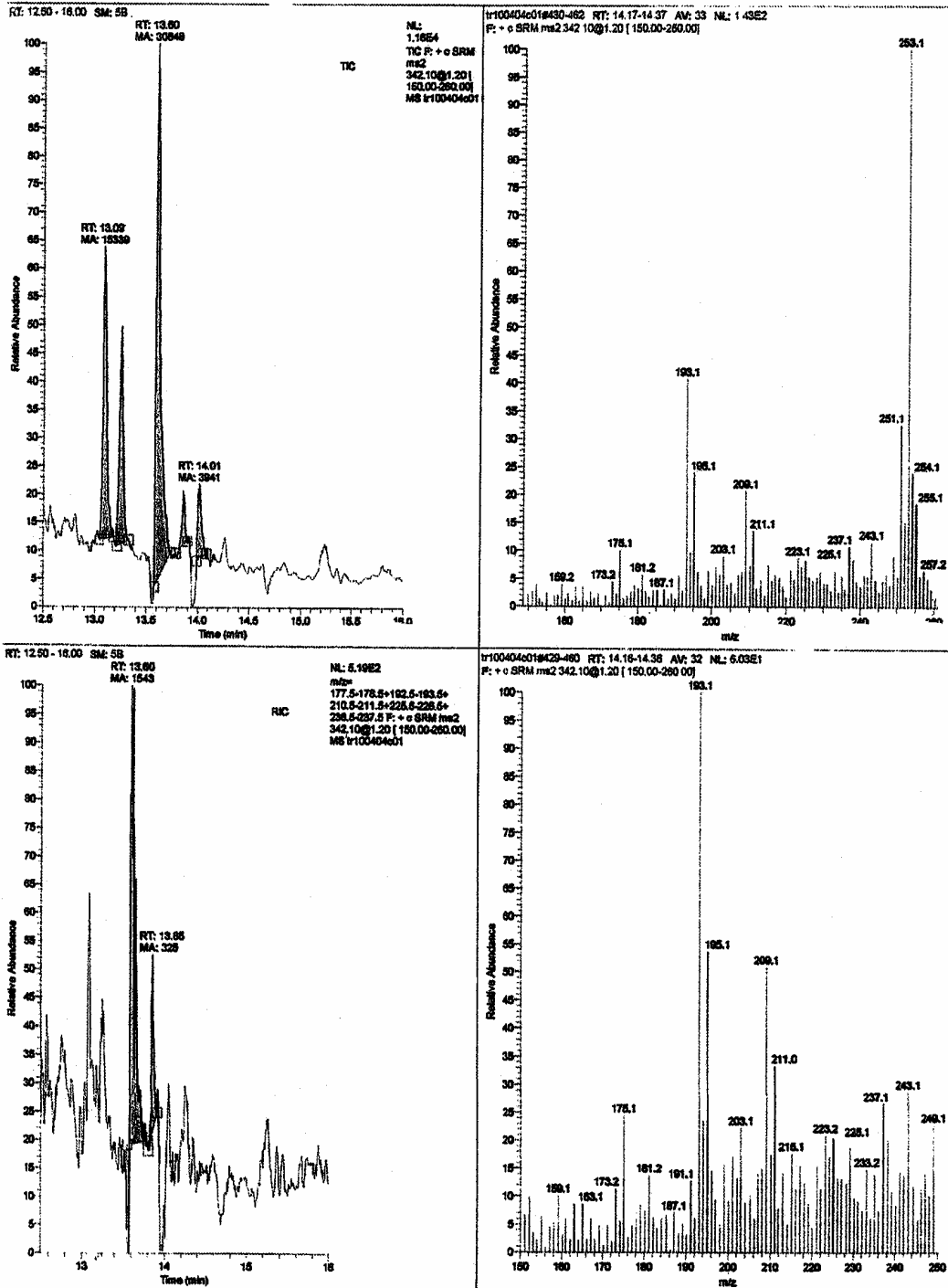
c. Total Ion Chromatogram (TIC) of 5 ppb  $\alpha$ -trenbolone fortified Bovine Liver (top trace) and Reconstructed Ion Chromatogram (RIC) - bottom trace.



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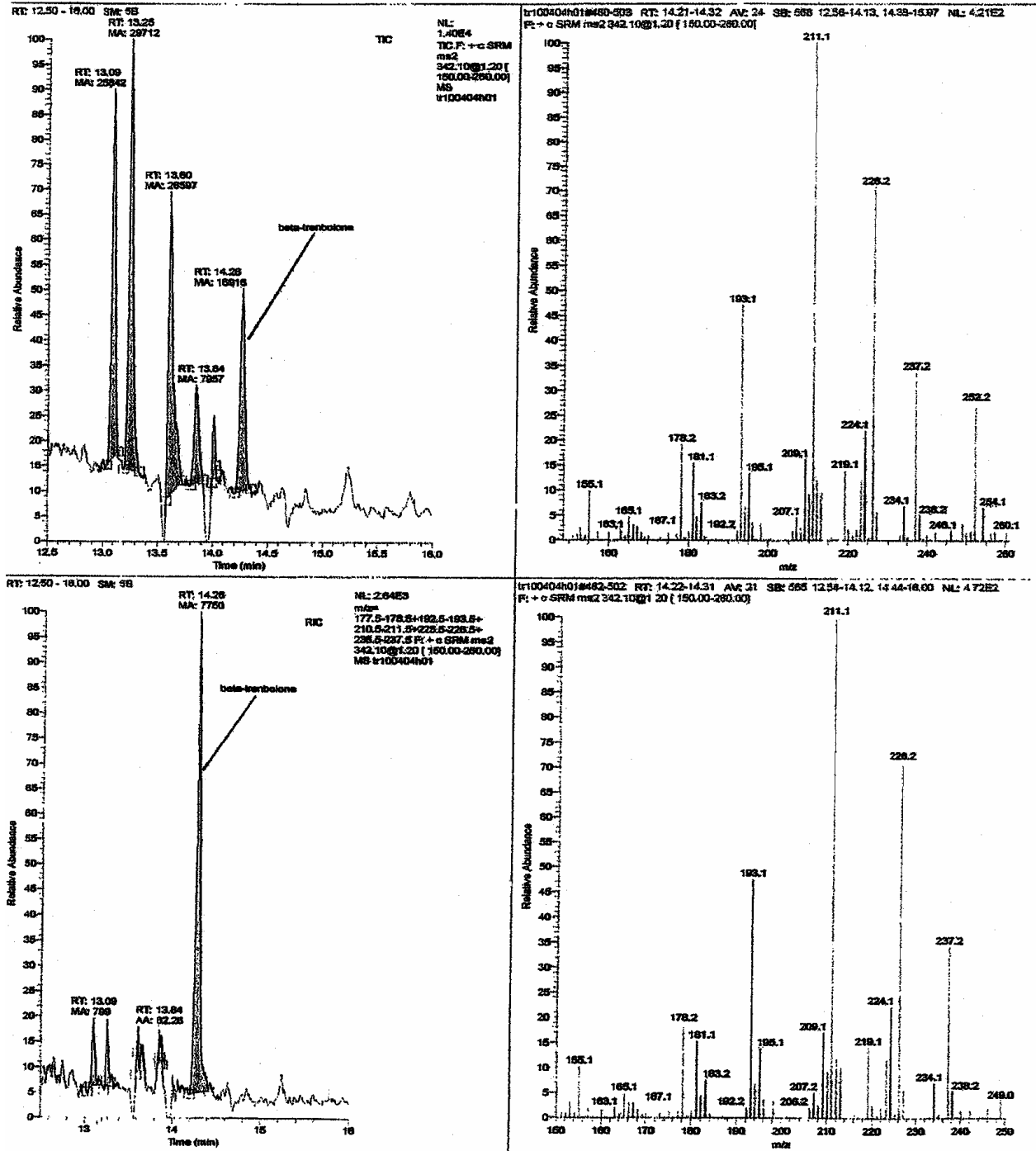
d. Chromatogram (TIC) of blank beef muscle (top trace) and Reconstructed Ion Chromatogram (RIC) - bottom trace.



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e. Total Ion Chromatogram (TIC) of 5 ppb  $\beta$ -trenbolone fortified beef muscle (top trace) and Reconstructed Ion Chromatogram (RIC) - bottom trace.



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