

This SOP has been Approved by the Laboratory Director, and Acknowledged by the Laboratory Supervisor and the Quality Assurance Officer.

DETECTION, QUANTIFICATION AND CONFIRMATION OF EIGHT ANABOLIC STEROIDS IN EQUINE PLASMA BY LC/TSQ-MS/MS

DEVELOPED BY

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DETECTION, QUANTIFICATION AND CONFIRMATION OF EIGHT ANABOLIC STEROIDS IN EQUINE PLASMA BY LC/TSQ-MS/MS

I. INTRODUCTION

Anabolic steroids (AS) are synthetic substances related to the male sex hormones (androgens). AS promote the growth of skeletal muscle (anabolic effects) and the development of male sexual characteristics (androgenic effects). These compounds are used in treating delayed puberty, select impotence, and wasting of the body caused by some diseases. AS are abused by bodybuilders, weightlifters and other athletes in human and equine athletes, and has been prohibited by the International Olympic Committee. Its use in horseracing has also been prohibited by the Association of Racing Commissioners' International, and they are, therefore, included in the list of prohibited substances in the horse. GC-MS is the technique commonly used by forensic laboratories for screening and confirmation of AS. Urine samples from donor athletes are analyzed and the metabolites of AS are monitored. However, GC-MS methods are limited by insensitivity, tedious procedure for derivatization of the metabolites and complication in characterization and confirmation of the metabolites that are very similar in molecular structures to one another. This SOP describes a sensitive LC-MS/MS method for screening, quantification and confirmation of eight major AS (boldenone, nandrolone, stanozolol, tetrahydrogestrinone also known as THG, testosterone, normethandrolone, methandrostenolone, and trenbolone) in equine plasma. The select AS in this study were chosen based on their tendency for use and abuse in equine athletes (personal communication with Dr. Pack at Penn National race Track in PA, USA, 2003).

II. SCOPE

This standard operating procedure will be limited to screening, quantification and confirmation of the eight anabolic steroids (Figure 1) in equine plasma samples. The scope of this work covers verifiable procedures to be used in quantifying and confirming the presence of the anabolic steroids in equine plasma. The limit to reporting a positive finding in a test sample to the Racing Commission is confirmation regardless of the concentration of AS in the sample.

III. PRINCIPLE OF METHODS

Anabolic steroids are neutral compounds and can be extracted with liquid-liquid extraction from equine plasma samples without pH adjustments. The extracts are reconstituted in LC sample solvent (mobile phase) and the analytes are identified and determined by liquid-chromatography integrated with tandem mass spectrometry operated under electrospray ionization (ESI) positive ion mode conditions. The multiple reaction monitoring (MRM) is used for detection, quantification and confirmation of the steroids. The concentration of the anabolic steroids is determined by the internal standard method using the chromatographic peak area ratio. Methenolone (Figure 1) is used as the internal standard. The limit of quantification (LOQ) for the steroids in equine plasma by this method is 25 pg/mL. The confirmable concentration of the anabolic steroids in equine plasma is 25 pg/mL for boldenone, 50 pg/mL for normethandrolone, nandrolone and methandrostenolone, and 100 pg/mL for testosterone, THG, trenbolone and stanozolol.

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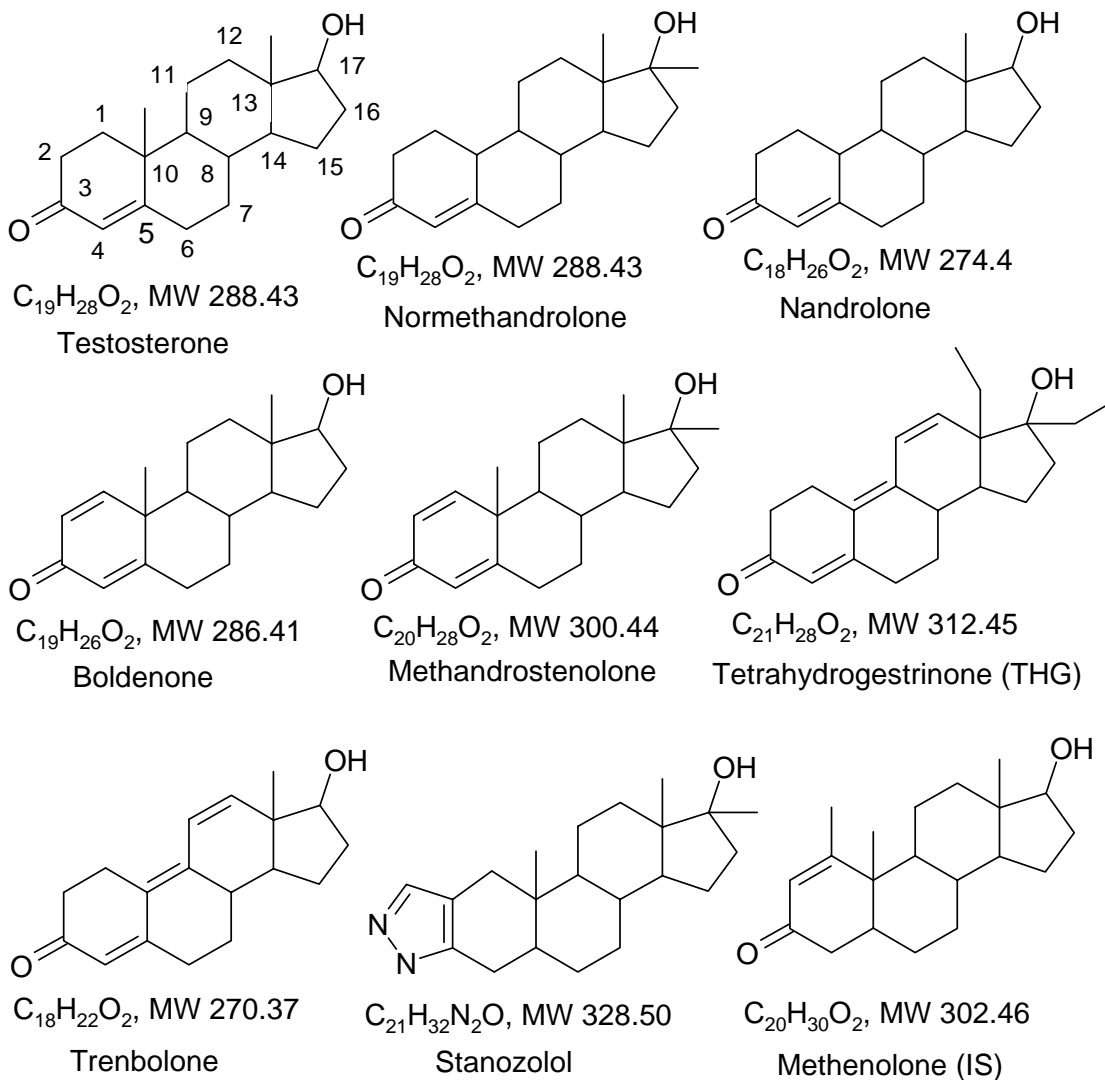


Figure 1. Chemical Structures of the Anabolic Steroids in this SOP

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IV. REAGENTS

- A. Methanol, HPLC grade (Cat. No. A 452-4, Fisher Scientific. Phone: 770-871-4500).
- B. Water, HPLC grade (Cat. No. W5-4, Fisher Scientific).
- C. Methyl tert-butyl ether, HPLC grade (Cat. No. E-127-4, Fisher Scientific)
- D. Ammonium formate, (Cat. No. A666-500, Fisher Scientific)
- E. Formic Acid, reagent A.C.S. (Cat. No. 200-5791, Sigma. Phone: 1-800-325-3010)

V. SOLUTIONS

A. Ammonium Formate Buffer (1.0 M, pH 3.4)

- 1. Reagents
 - a) Ammonium formate (HCOONH_4)
 - b) Formic acid (HCOOH , 88%)
 - c) Water, HPLC grade
- 2. Procedure
 - a) Weigh 6.3 grams of ammonium formate and dissolve it in 80 mL water (HPLC grade) in a 200 mL beaker.
 - b) Add 4.3 mL formic acid (88%). Adjust the pH to 3.4 with formic acid and ammonium hydroxide.
 - c) Transfer the solution into a 100 mL volumetric flask, and bring to 100 mL total volume by adding water (HPLC grade).
- 3. Storage Requirements
 - a) Store in a glass container at 4 °C (refrigerator).
 - b) Discard 6 months after date of preparation

B. HPLC Solvent A --- Ammonium Formate (2.0 mM, pH 3.4)

- 1. Reagents
 - a) 1.0 M Ammonium Formate
 - b) Water, HPLC grade
- 2. Procedure
 - a) Add 1000 mL of water to a liter glass container.
 - b) Add 2 mL of ammonium formate (1.0 M, pH 3.4). Mix.
- 3. Storage Requirements
 - a) Store at room temperature in a glass bottle.
 - b) Discard 3 months after date of preparation

VI. PRIMARY DRUG AND INTERNAL STANDARD REFERENCE MATERIALS

- A. Boldenone, FW: 286.41 (Cat. No. A0200-000, Steraloids, Rhode Island, Phone: 401-848-5422).
- B. Nandrolone, FW: 274.40 (Cat. No. E4050-000, Steraloids, Rhode Island).
- C. Stanozolol, FW: 328.50 (Cat. No. A2120-000, Steraloids, Rhode Island).
- D. Testosterone, FW: 288.43 (Cat. No. A6950-000, Steraloids, Rhode Island).

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- E. Methandrostenolone, FW: 300.44 (Cat. No. A0130-040, Steraloids, Rhode Island).
- F. Normethandrolone, FW: 288.43 (Cat. No. E3900-000, Steraloids, Rhode Island).
- G. Trenbolone, FW: 270.37 (Cat. No. E3170-000, Steraloids, Rhode Island).
- H. THG, FW: 312.42, kindly donated by Dr. Thomas Tobin at the University of Kentucky, KY, USA, (Phone: 859-257-4757 ext 8-1092).
- I. Methenolone, internal standard, FW: 302.46 (Cat. No. A4420-000, Steraloids, Rhode Island).

Obtain these materials from the QAO. Record accession of these materials on the pharmacy log sheet.

VII. PREPARATION OF PRIMARY REFERENCE STOCK SOLUTIONS

A. Boldenone

- 1. Materials
 - a) Boldenone, (Cat. No. A0200-000, Steraloids, Rhode Island).
 - b) Methanol (HPLC grade)
- 2. Procedure
 - a) Weigh between 3 and 5 mg (X.xx mg) of boldenone into a 7 mL amber glass vial.
 - b) Add certain volume (Volume Y.yy = X.xx in mL) of methanol to the vial. Cap and mix until boldenone is completely dissolved in methanol.
 - c) The resulting concentration of boldenone is 1.00 mg/mL.
- 3. Storage Requirements
 - a) Store at approximately 4 °C (refrigerator).
 - b) Discard 6 months after preparation.

B. Nandrolone

- 1. Materials
 - a) Nandrolone, (Cat. No. E4050-000, Steraloids, Rhode Island).
 - b) Methanol (HPLC grade).
- 2. Procedure
 - a) Weigh between 3 and 5 mg (X.xx mg) of nandrolone into a 7 mL amber glass vial.
 - b) Add certain volume (Volume Y.yy = X.xx in mL) of methanol to the vial. Cap and mix until nandrolone is completely dissolved in methanol.
 - c) The resulting concentration of nandrolone is 1.00 mg/mL.
- 3. Storage Requirements
 - a) Store at approximately 4 °C (refrigerator).
 - b) Discard 6 months after preparation.

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C. Stanozolol

1. Materials

- a) Stanozolol, (Cat. No. A2120-000, Steraloids, Rhode Island).
- b) Methanol (HPLC grade)

2. Procedure

- a) Weigh between 3 and 5 mg (X.xx mg) of stanozolol into a 7 mL amber glass vial.
- b) Add certain volume (Volume Y.yy = X.xx in mL) of methanol to the vial. Cap and mix until stanozolol is completely dissolved in methanol.
- c) The resulting concentration of stanozolol is 1.00 mg/mL.

3. Storage Requirements

- a) Store at approximately 4 °C (refrigerator).
- b) Discard 6 months after preparation.

D. Testosterone

1. Materials

- a) Testosterone, (Cat. No. A6950-000, Steraloids, Rhode Island).
- b) Methanol (HPLC grade)

2. Procedure

- a) Weigh between 3 and 5 mg (X.xx mg) of testosterone into a 7 mL amber glass vial.
- b) Add certain volume (Volume Y.yy = X.xx in mL) of methanol to the vial. Cap and mix until testosterone is completely dissolved in methanol.
- c) The resulting concentration of testosterone is 1.00 mg/mL.

3. Storage Requirements

- a) Store at approximately 4 °C (refrigerator).
- b) Discard 6 months after preparation.

E. Methandrostenolone

1. Materials

- a) Methandrostenolone, (Cat. No. A0130-040, Steraloids, Rhode Island).
- b) Methanol (HPLC grade)

2. Procedure

- a) Weigh between 3 and 5 mg (X.xx mg) of methandrostenolone into a 7 mL amber glass vial.
- b) Add certain volume (Volume Y.yy = X.xx in mL) of methanol to the vial. Cap and mix until methandrostenolone is completely dissolved in methanol.
- c) The resulting concentration of methandrostenolone is 1.00 mg/mL.

3. Storage Requirements

- a) Store at approximately 4 °C (refrigerator).

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- b) Discard 6 months after preparation.

F. Normethandrolone

1. Materials

- a) Normethandrolone (Cat. No. E3900-000, Steraloids, Rhode Island).
- b) Methanol (HPLC grade)

2. Procedure

- a) Weigh between 3 and 5 mg (X.xx mg) of normethandrolone into a 7 mL amber glass vial.
- b) Add certain volume (Volume Y.yy = X.xx in mL) of methanol to the vial. Cap and mix until normethandrolone is completely dissolved in methanol.
- c) The resulting concentration of normethandrolone is 1.00 mg/mL.

3. Storage Requirements

- a) Store at approximately 4 °C (refrigerator).
- b) Discard 6 months after preparation.

G. Trenbolone

1. Materials

- a) Trenbolone, FW: 270.37 (Cat. No. E3170-000, Steraloids, Rhode Island).
- b) Methanol (HPLC grade)

2. Procedure

- a) Weigh between 3 and 5 mg (X.xx mg) of trenbolone into a 7 mL amber glass vial.
- b) Add certain volume (Volume Y.yy = X.xx in mL) of methanol to the vial. Cap and mix until trenbolone is completely dissolved in methanol.
- c) The resulting concentration of trenbolone is 1.00 mg/mL.

3. Storage Requirements

- a) Store at approximately 4 °C (refrigerator).
- b) Discard 6 months after preparation.

H. THG

1. Materials

- a) THG, FW: 312.42, kindly donated by Dr. Thomas Tobin at the University of Kentucky.
- b) Methanol (HPLC grade)

2. Procedure

- a) Weigh between 3 and 5 mg (X.xx mg) of THG into a 7 mL amber glass vial.
- b) Add certain volume (Volume Y.yy = X.xx in mL) of methanol to the weighed drug in the vial. Cap and mix until THG is completely dissolved in methanol.
- c) The resulting concentration of THG is 1.00 mg/mL.

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3. Storage Requirements

- a) Store at approximately 4 °C (refrigerator).
- b) Discard 6 months after preparation.

I. Methenolone

1. Materials

- a) Methenolone, internal standard, FW: 302.46 (Cat. No. A4420-000, Steraloids, Rhode Island).
- b) Methanol (HPLC grade)

2. Procedure

- a) Weigh between 3 and 5 mg (X.xx mg) of methenolone into a 7 mL amber glass vial.
- b) Add certain volume (Volume Y.yy = X.xx in mL) of methanol to the vial. Cap and mix until methenolone is completely dissolved in methanol.
- c) The resulting concentration of methenolone is 1.00 mg/mL.

3. Storage Requirements

- a) Store at approximately 4 °C (refrigerator).
- b) Discard 6 months after preparation.

Complete the Balance Use Log and QA Primary Reference Standard Log for this process.

Label the primary reference stock solutions with QA Primary Reference Log SR# (i.e. SR# xxx) and Primary Reference Powder Designation (i.e. R-NAND-1).

VIII. PREPARATION OF SECONDARY REFERENCE STOCK SOLUTIONS

A. Standard Working Solution of Mixture of Boldenone, Nandrolone, Stanozolol, Testosterone, Methandrostenolone, Normethandrolone, THG and Trenbolone

1. Materials

- a) 1.00 mg/mL of boldenone stock solution.
- b) 1.00 mg/mL of nandrolone stock solution.
- c) 1.00 mg/mL of stanozolol stock solution.
- d) 1.00 mg/mL of testosterone stock solution.
- e) 1.00 mg/mL of methandrostenolone stock solution.
- f) 1.00 mg/mL of normethandrolone stock solution.
- g) 1.00 mg/mL of THG stock solution.
- h) 1.00 mg/mL of trenbolone stock solution.
- i) Methanol (HPLC grade).

2. Procedure

- a) Prepare secondary stock solutions of each anabolic steroid according to Table 1.
- b) Prepare working solutions of the mixture of anabolic steroids of different concentrations according to Table 2.

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3. Storage Requirements

- a). Store at approximately 4 °C (refrigerator).
- b). Discard 3 months after preparation.

Table 1. Preparation of Secondary Stock Solution of Each Anabolic Steroid

Target Con., µg/mL	Stock Solution used, µg/mL	Vol. Added, µL	Vol. of MeOH, mL
100	1000	500	4.50
10	100	500	4.50

Table 2. Preparation of Working Solution of the Mixture of Six Anabolic Steroids

Target concn. of 6 steroids ng/mL	Total volume mL	Solvent	St. of Bolden. µg/mL	St. of Nandr. µg/mL	St. of Stanozol µg/mL	St. of Testost. µg/mL	St. of Normeth. µg/mL	St. of Methandr. µg/mL	St. of THG µg/mL	St. of Trenb. µg/mL	Vol.of each ST mL	Vol. of MeOH mL
100	10	Methanol	10	10	10	10	10	10	10	10	0.10	9.2
10	10	Methanol	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	1.0 total	9.0
1.0	10	Methanol	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	1.0 total	9.0

St = stock solution

B. Methenolone Working Solution (IS, 50 ng/mL)

1. Materials

- a) 1.0 mg/mL of methenolone stock solution
- b) Methanol

2. Procedure

- a) Transfer 5.0 mL of methanol into a 7-mL glass vial.
- b) Take out 50 µL of methanol.
- c) Add 50 µL of 1.0 mg/mL of methenolone standard stock solution. Mix.
- d) The concentration of trenbolone is 10 µg/mL.
- e) Transfer 15.0 mL of methanol into another 15-mL glass vial.
- f) Take out 75 µL of methanol.
- g) Add 75 µL of 10 µg/mL of methenolone standard solution.
- h) The resulting concentration of trenbolone is 50 ng/mL.

3. Storage Requirements

- a) Store at approximately 4 °C (refrigerator).
- b) Discard 3 months after preparation.

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IX. QC WORKING SOLUTIONS

A. Anabolic steroid QC Working Solution

1. Materials

- a) 1.00 mg/mL of boldenone stock solution.
- b) 1.00 mg/mL of nandrolone stock solution.
- c) 1.00 mg/mL of stanozolol stock solution.
- d) 1.00 mg/mL of testosterone stock solution.
- e) 1.00 mg/mL of methandrostenolone stock solution.
- f) 1.00 mg/mL of normethandrolone stock solution.
- g) 1.00 mg/mL of THG stock solution.
- h) 1.00 mg/mL of trenbolone stock solution.
- i) Methanol (HPLC grade).

2. Procedure

- a) Prepare secondary stock solutions of each anabolic steroid according to Table 1.
- b) Prepare working solutions of the mixture of anabolic steroids of different concentrations according to Table 2.

3. Storage Requirements

- a) Store at approximately 4 °C (refrigerator)
- b) Discard 3 months after preparation.

X. MATERIALS

- A. Balance (Mettler AT 261 Delta range, Mettler-Toledo Inc.)
- B. 16 × 100 mm test tubes.
- C. 16 × 125 mm screw-top test tubes.
- D. Screw caps
- E. Test tube rack
- F. Pipettes and tips.
- G. Vortex mixer (Scientific Industries, Inc.)
- H. Rotorack (Speci-Mix, Thermolyne)
- I. IEC HN-SII Centrifuge (International Equipment Company)
- E. Sample Concentrator (Dri-Block DB-3, Techne, New Jersey; Phone: 1-800-225-9243)
- F. 2 mL autosampler vials
- G. 200 uL Insert (Target PP Polyspring, National Scientific Company; ordered from Fisher Scientific)
- H. Eye protection
- I. Gloves

XI. MATRIX

Equine Plasma.

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XII. VOLUME OF MATRIX FOR ANALYSIS

1.0 mL

XIII. CONTROL SAMPLES

A. Negative Control Sample

1. Equine plasma samples previously demonstrated by LC-MS to be negative for the presence of detectable anabolic steroids.
2. Store control samples at approximately -20°C

B. Positive Control Sample

1. Equine plasma samples supplemented with anabolic steroids at concentrations of 50, 500 and 5000 pg/mL.

XIV. SAMPLE REQUIREMENTS FOR ANALYSIS

A. Calibrators

1. Prepare a set of calibrators for analysis of plasma samples.
2. Calibrator concentrations as designated in Table 3.
3. Prepare plasma calibrators using negative (control) plasma and anabolic steroid standard working solutions as described in Section VIII.

B. Negative (control) sample

1. Designate **plasma NC or plasma blank**.
2. Prepare negative (control) sample from negative (control) plasma.

C. Positive control samples

1. Designate **plasma QC (or PC)1.....QC (or PC)n**.
2. Prepare positive control samples as described in Table 4.

D. Mobile phase blank

1. Designate **MB1.....MBn**

E. Test samples designated to use the date of which the sample is analyzed and raw data file designated to use sequence number.

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Table 3. Preparation of Plasma Calibrators for the anabolic steroids

Target Conc. of each anabolic steroid (pg/mL)	Working mixture Solution (ng/mL)	Vol. of Spiked Working Solution (µL)	Volume of Plasma (mL)	Vol. of Spiked 50 ng/mL of IS
25	1.0	25	1.0	20
50	1.0	50	1.0	20
100	1.0	100	1.0	20
250	10	25	1.0	20
500	10	50	1.0	20
1000	10	100	1.0	20
2500	100	25	1.0	20
5000	100	50	1.0	20
10000	100	100	1.0	20

Table 4. Preparation of Plasma Positive Control (QC) Samples for the Anabolic Steroids

Target Conc. of each anabolic steroid (pg/mL)	QC Working Solution of the mixture (ng/mL)	Vol. of Spiked QC Working Solution (µL)	Volume of Plasma (mL)	Vol. of Spiked 50 ng/mL of IS
50	1.0	50	1.0	20
50	1.0	50	1.0	20
500	10	50	1.0	20
500	10	50	1.0	20
5000	100	50	1.0	20
5000	100	50	1.0	20

XV. PLASMA CALIBRATOR AND SAMPLE PREPARATION

A. Calibrator and Sample Preparation for the Anabolic Steroids.

1. Label 16 × 125 mm test tubes.
2. All samples are prepared in labeled tubes as per Table 5.
3. Vortex for 5-10 seconds to mix the contents of each tube.

Table 5. Preparation of Plasma Samples for the Anabolic Steroid Analysis

Items	Blank	Blank+IS	Sample
Blank Plasma (mL)	1.0	1.0	N/A
IS Solution (µL)	0	20	20
Plasma Samples (mL)	N/A	N/A	1.0

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B. Sample Extraction by Liquid-Liquid Extraction

Safety Requirements: eye protection

1. Take 1.0 mL aliquot of each concentration sample into individual screw-top test tube.
2. Add 5 mL of methyl tert-butyl ether (MTBE) into each tube, cap all screw-top tubes tightly with screw caps, and shake on a rotorack for 4 minutes.
3. Centrifuge at 2,500 ~ 3,000 rpm (839 ~ 1,409 g) for 5 minutes.
4. Decant the (top) organic layer into a labeled fresh test tube for each sample.
5. Bring the extracts in test tubes to dryness in a fume hood at 50 °C under a stream of nitrogen.
6. Remove test tubes from the drying block, place in a rack, and allow to cool to room temperature.
7. Reconstitute the residues with 100 µL of 60% methanol in ammonium formate (2 mM, pH 3.4)
8. Transfer the above solution into a 200 µL insert and load in the autosampler vials. All the samples are now ready for LC/MS/MS analysis.

XVI. LIQUID CHROMATOGRAPHIC/MASS SPECTRAL IDENTIFICATION AND QUANTITATION OF ANABOLIC STEROIDS

A. Instrumentation

1. ThermoFinnigan TSQ Quantum AM triple quadrupole mass spectrometer with Xcalibur V1.3 for system control and data acquisition and processing (AM = Accurate Mass).
2. Surveyor MS quaternary HPLC pump, autosampler, column compartment and on-line degasser.

B. HPLC conditions

1. HPLC Column
 - a) Type: Ace C8 Analytical Column (Part No. ACE-122-0502, Mac-Mod, Chadds Ford, PA; Phone 1-800-441-7508).
 - b) Dimension: 2.1 × 50 mm
 - c) Particle size: 5 Micron
 - d) Temperature: ambient
2. LC Guard Column
 - a) Type: Ace C8 (Part No. ACE-122-0102GD, Mac-Mod).
 - b) Dimension: 2.1 × 12.5 mm
 - c) Particle size: 5 micron
 - d) Temperature: ambient
3. Mobile Phase
 - a) Mobile phase A: 2.0 mM HCOONH₄ in H₂O (pH 3.4)
 - b) Mobile phase B: Methanol
4. Mobile phase and flow rate gradient are shown in Table 6.

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Table 6. Gradients of LC mobile phase and flow rate

LC Run time (min)	Formate buffer ^a (%)	Methanol (%)	Flow rate (µL/min)
0	40	60	300
4.0	40	60	300
8.0	10	90	300
8.1	10	90	300
8.2	40	60	400
10.4	40	60	400
10.5	40	60	300

^a Ammonium formate, 2 mM, pH 3.4.

5. Injection Volume: 20 µL.

C. Mass Spectrometric Conditions

1. Ionization mode

- a) Electrospray ionization (ESI)
- b) Positive ion mode

2. ESI source settings

- a) Sheath gas (nitrogen) pressure: 49 (units)
- b) Auxiliary gas flow: 6.0
- c) Spray voltage: 4500 V (volts)
- d) Capillary Temperature: 300 °C.
- e) Source CID: 20 V

3. MRM acquisition parameters are shown in Table 7.

Table 7. Parameters for MRM acquisition of testosterone, normethandrolone, nandrolone, boldenone, methandrostenolone, THG, trenbolone and stanozolol

	Testosterone	Normethan-drolone	Nandrolone	Boldenone	Methandro- stenolone	THG	Trenbolone	Stanozolol
Retention time (min)	4.46	4.57	3.39	2.99	3.92	6.93	2.98	7.89
Ion transition (<i>m/z</i>) for confirmation (collision energy, V)	289 → 253, 109, 97 (16)	289 → 253, 231, 213 (18)	275 → 239, 145, 109 (17)	287 → 173, 135, 121 (15)	301 → 173, 149, 121 (15)	313 → 266, 241, 239 (15)	271 → 227, 199, 183 (20)	329 → 329, 121, 107 (40)
Ion transition (<i>m/z</i>) for quantification (collision energy, V)	289 → 109 (16)	289 → 213 (18)	275 → 109 (17)	287 → 121 (15)	301 → 121 (15)	313 → 266 (15)	271 → 199 (20)	329 → 329 (40)

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D. Sample list setup for anabolic steroid analysis

1. Sample solvent blank
2. Blank plasma (QC negative control)
3. Blank plasma + IS (1000 pg/mL)
4. QC plasma sample (50 pg/mL for each anabolic steroid)
5. QC plasma sample (500 pg/mL for each anabolic steroid)
6. QC plasma sample (5000 pg/mL for each anabolic steroid)
7. Sample solvent blank
8. Calibrator series 1 (10 pg/mL)
9. Calibrator series 2 (25 pg/mL)
10. Calibrator series 3 (50 pg/mL)
11. Calibrator series 4 (100 pg/mL)
12. Calibrator series 5 (250 pg/mL)
13. Calibrator series 6 (500 pg/mL)
14. Calibrator series 7 (1000 pg/mL)
15. Calibrator series 8 (2500 pg/mL)
16. Calibrator series 9 (5000 pg/mL)
17. Calibrator series 10 (10000 pg/mL)
18. Sample solvent blank
19. Sample 1, replicate 1
20. Sample 1, replicate 2
21. Sample 1, replicate 3
22. Sample solvent blank
23. Sample 2, replicate 1
24. Sample 2, replicate 2
25. Sample 2, replicate 3
26. Sample solvent blank
27. QC plasma sample (50 pg/mL for each anabolic steroid)
28. QC plasma sample (500 pg/mL for each anabolic steroid)
29. QC plasma sample (5000 pg/mL for each anabolic steroid)
30. Sample solvent blank

There should be a mobile phase blank in between sample, calibrator and QC.

E. Criteria for Identification of Anabolic Steroids in Equine Plasma Extracts

- a) The confirmation of anabolic steroids is performed using the specific ion transitions for each anabolic steroid and the intensity ratio of different product ions of each anabolic steroid. The qualifying ions and the ratio for each analyte are listed in Table 8. These values are for reference. In practice, the intensity ratio of product ions for a positive sample should be within $\pm 25\%$ of the average value of those from the calibrators and QCs analyzed along with the positive sample in the same batch.

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- b) Under LC-MS/MS analytical conditions, all the product ions of an anabolic steroid must be recognized at retention time within ± 0.2 min of that of an authentic standard analyzed under identical conditions.

Table 8. Intensity ratios of major product ions of each anabolic steroid at different concentrations for confirmation

Concentration (pg/mL)		25	50	100	250	500	1000	2500	50000	100000	Mean	SD
Testosterone	<i>m/z</i> 109/97			100	86	76	89	85	82	87	87	8
	<i>m/z</i> 253/97			19	24	20	21	23	22	23	22	2
Normethandrolone	<i>m/z</i> 231/213		77		76	70	67	77	77	77	74	4
	<i>m/z</i> 253/213		61		71	68	71	82	75	77	74	8
Nandrolone	<i>m/z</i> 145/109		50		52	56	64	58	60	61	57	5
	<i>m/z</i> 239/109		87		78	79	83	81	75	80	80	4
Boldenone	<i>m/z</i> 173/121	32	31	25	28	28	29	28	28	28	29	2
	<i>m/z</i> 135/121	56	60	51	54	53	55	54	54	53	54	3
Methandrostenolone	<i>m/z</i> 149/121		92	86	89	93	90	86	90	89	89	2
	<i>m/z</i> 173/121		22	18	19	20	19	20	20	20	20	1
THG	<i>m/z</i> 239/241			51	60	49	52	49	54	51	52	4
	<i>m/z</i> 266/241			35	32	34	37	35	33	34	33	4
Trenbolone	<i>m/z</i> 183/199			36	22	33	28	28	27	27	29	5
	<i>m/z</i> 227/199			49	43	55	48	47	46	46	47	4
Stanozolol	<i>m/z</i> 107/329			21	21	22	22	22	22	23	22	3
	<i>m/z</i> 121/329			27	26	26	27	27	27	28	27	3

F. Criteria for Anabolic Steroid Quantitation

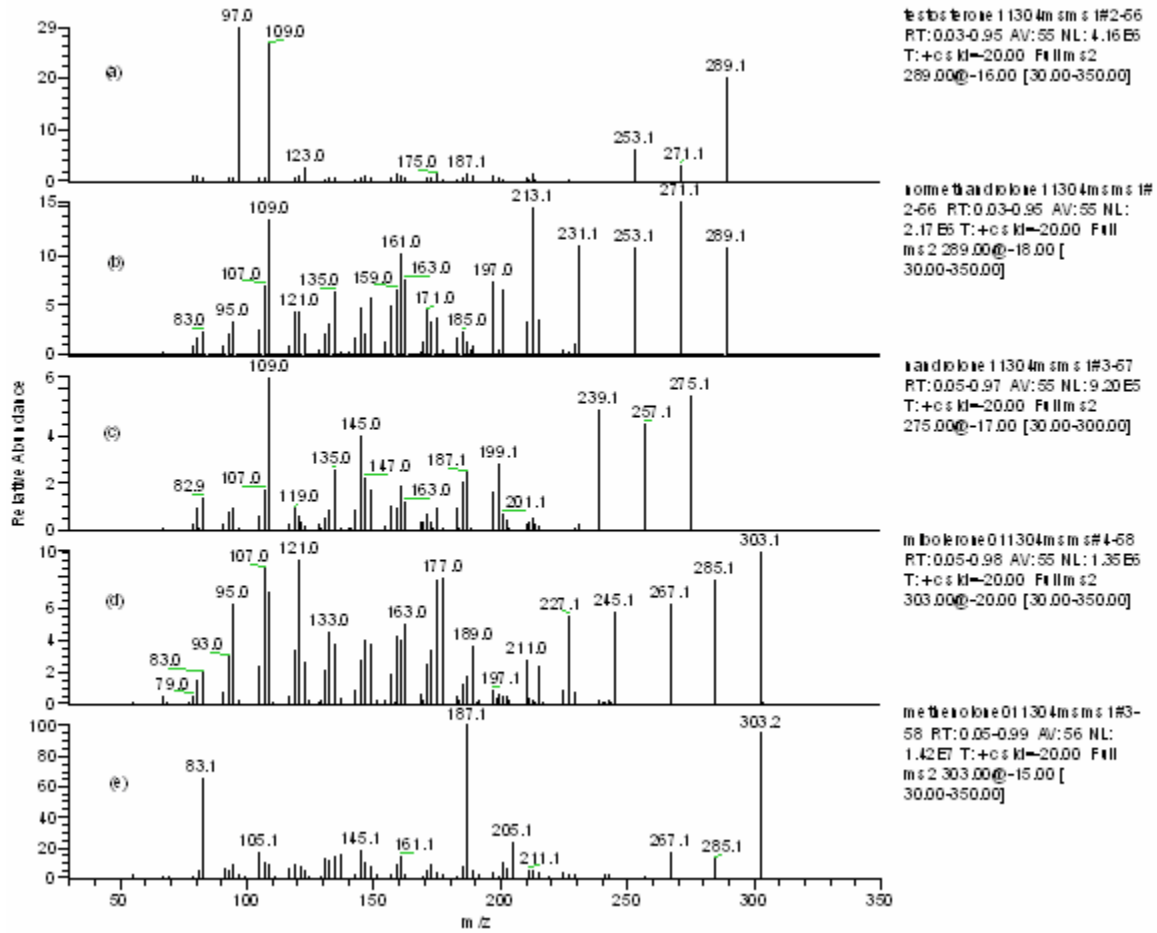
1. Determination of Anabolic Steroids

- The ion transition used for quantification of testosterone is “*m/z* 289 → 109”.
- The ion transition used for quantification of normethandrolone is “*m/z* 289 → 213”.
- The ion transition used for quantification of nandrolone is “*m/z* 275 → 109”.
- The ion transition used for quantification of boldenone is “*m/z* 287 → 121”.
- The ion transition used for quantification of methandrostenolone is “*m/z* 301 → 121”.
- The ion transition used for quantification of THG is “*m/z* 313 → 266”.
- The ion transition used for trenbolone is “*m/z* 271 → 199”.
- The ion transition used for quantification of stanozolol is “*m/z* 329 → 329”.
- The ion transition used for methenolone (internal standard) is “*m/z* 303 → 187”.
- Plot the peak area ratios of the quantifying ions for each calibrator versus the concentration of each anabolic steroid in the calibrator (internal calibration). Use the Xcaliber software’s Quantification function to perform calibration and quantitation. Print the compound summary quantification report and calibration curve (Figure 9). The correlation should be greater than 0.98.
- Examine the reported concentrations for all samples. The accuracy of concentrations for QC samples should be between 80% and 120 %.

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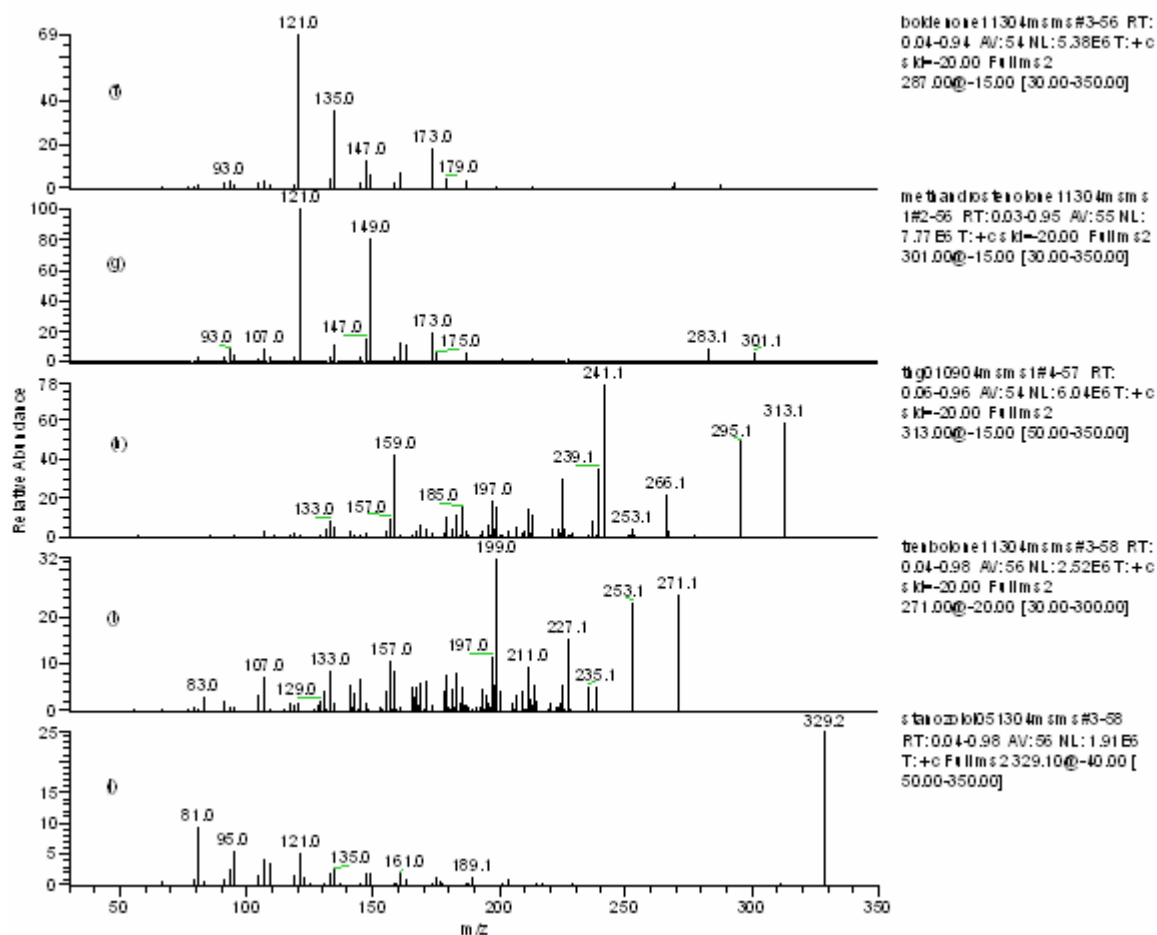


Figure 2. MS/MS spectra of testosterone (a), normethandrolone (b), nandrolone (c), mibolerone (d), methenolone (e), boldenone (f), methandrolone (g), THG (h), trenbolone (i), and stanozolol (j). Collision energy was 16 V for testosterone, 18 V for normethandrolone, 17 V for nandrolone, 20 V for mibolerone and trenbolone, 15 V for methenolone, boldenone, methandrostenolone and THG, 40 V for stanozolol.

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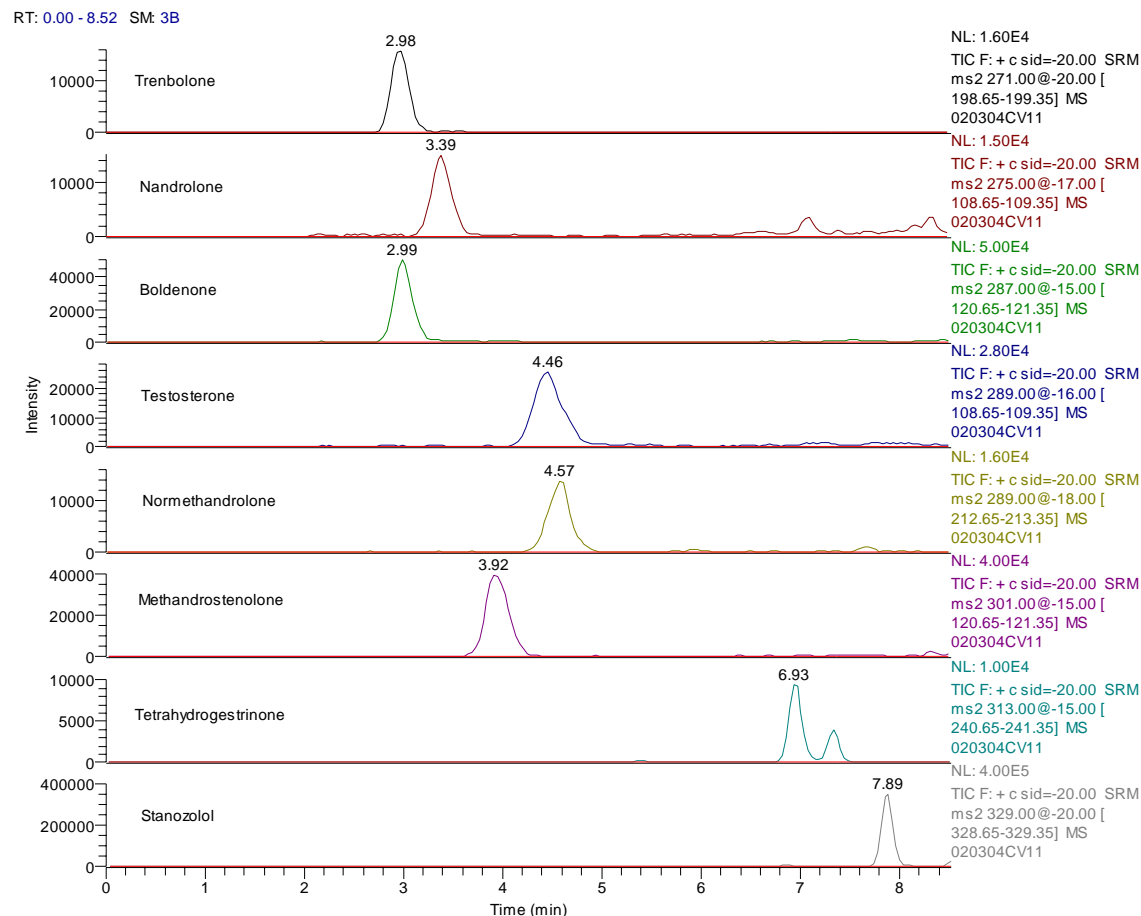


Figure 3. LC-MS/MRM chromatograms showing the peaks of testosterone, normethandrolone, nandrolone, boldenone, methandrostenolone, THG, trenbolone and stanozolol that were spiked into equine plasma (250 pg/ml of each). Not shown in the chromatograms are methenolone (IS) at retention time of 6.03 min and mibolerone at retention time of 5.6 min.

XVII. CRITERIA FOR REPORTING A SAMPLE AS A POSITIVE FINDING FOR THE PRESENCE OF SELECT ANABOLIC STEROID

Report a test sample as a positive per this standard operating procedure for a select anabolic steroid if the sample contains one of the anabolic steroids greater than a set concentration with 95 % Confidence Interval, and all of the following criteria are met:

- The test sample contains an anabolic steroid according to the chromatographic and product ion ratio criteria described in XVI (E).

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- The limit of quantitation of an anabolic steroid in the test sample is better than 25 pg/mL plasma (NOTE: in PA, confirmation not concentration is the only limiting criterion for a positive finding).
- The signal-to-noise ratio of the least abundant qualifying ion for an anabolic steroid in each replicate of the test sample is greater than 10.
- The concentration for confirmation of an anabolic steroid in the test sample is greater than a set value (25, 50 or 100 pg/mL depending on the AS; see page 1 under Principal of Method).

XVIII. MEASUREMENT UNCERTAINTY

Without inter-laboratory assessment of a developed method, the measurement uncertainty is estimated according to the following statements.

1. Method measurement uncertainty is established based on method validation.
2. The 95% confidence interval is expressed as “± Standard Deviation × coverage factor (k)” (SD×k) for both unknown determinations as well as threshold control values.
3. The measurement uncertainty coverage factor (k, for 95% confidence level) is expressed as the following values depending on the number of data points.

k= 2.0 for 20-50 data points

k= 2.3 for 10-19 data points

k= 2.5 for 4-9 data points

4. The 95% confidence level of a measurement of concentration should be expressed as “mean value ± (k×SD)”.

Examples of calculating measurement uncertainty are shown below.

Measurement Uncertainty for testosterone in plasma samples

Symbol	Source of Uncertainty	Value of Units	Distribution	Divisor	Standard Uncertainty	Degree of Freedom (n-1)	Other
U1	Intermediate precision	6.9	N	1	6.9	13	Testosterone 500 pg/ml
U2	Intermediate precision	3.7	N	1	3.7	13	Testosterone 5000 pg/ml
Combined Uncertainty		1: $(U_1^2)^{1/2} = 6.9$; 2: $(U_2^2)^{1/2} = 3.7$					
Expanded Uncertainty (k= 2.3, 10-19 data points)		1: $(6.9 \times 2.3) = 16\%$ 2: $(3.7 \times 2.3) = 9\%$, Testosterone = concentration ± 16%					

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Measurement Uncertainty for normethandrolone in plasma samples

Symbol	Source of Uncertainty	Value of Units	Distribution	Divisor	Standard Uncertainty	Degree of Freedom (n-1)	Other
U1	Intermediate precision	7.2	N	1	7.2	13	Normethandrolone 500 pg/ml
U2	Intermediate precision	5.1	N	1	5.1	13	Normethandrolone 5000 pg/ml
Combined Uncertainty		1: $(U_1^2)^{1/2} = 7.2$; 2: $(U_2^2)^{1/2} = 5.1$					
Expanded Uncertainty (k= 2.3, 10-19 data points)		1: $(7.2 \times 2.3) = 17\%$ 2: $(5.1 \times 2.3) = 12\%$, Normethandrolone = concentration $\pm 17\%$					

Measurement Uncertainty for nandrolone in plasma samples

Symbol	Source of Uncertainty	Value of Units	Distribution	Divisor	Standard Uncertainty	Degree of Freedom (n-1)	Other
U1	Intermediate precision	9.0	N	1	9.0	13	Nandrolone 500 pg/ml
U2	Intermediate precision	3.5	N	1	3.5	13	Nandrolone 5000 pg/ml
Combined Uncertainty		1: $(U_1^2)^{1/2} = 9.0$; 2: $(U_2^2)^{1/2} = 3.5$					
Expanded Uncertainty (k= 2.3, 10-19 data points)		1: $(9.0 \times 2.3) = 21\%$ 2: $(3.5 \times 2.3) = 8\%$, Nandrolone = concentration $\pm 21\%$					

Measurement Uncertainty for boldenone in plasma samples

Symbol	Source of Uncertainty	Value of Units	Distribution	Divisor	Standard Uncertainty	Degree of Freedom (n-1)	Other
U1	Intermediate precision	6.3	N	1	6.3	13	Boldenone 500 pg/ml
U2	Intermediate precision	4.2	N	1	4.2	13	Boldenone 5000 pg/ml
Combined Uncertainty		1: $(U_1^2)^{1/2} = 6.3$; 2: $(U_2^2)^{1/2} = 4.2$					
Expanded Uncertainty (k= 2.3, 10-19 data points)		1: $(6.3 \times 2.3) = 14\%$ 2: $(4.2 \times 2.3) = 10\%$, Boldenone = concentration $\pm 14\%$					

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Measurement Uncertainty for methandrostenolone in plasma samples

Symbol	Source of Uncertainty	Value of Units	Distribution	Divisor	Standard Uncertainty	Degree of Freedom (n-1)	Other
U1	Intermediate precision	6.6	N	1	6.6	13	Methandr. 500 pg/ml
U2	Intermediate precision	4.1	N	1	4.1	13	Methandr. 5000 pg/ml
Combined Uncertainty		1: $(U_1^2)^{1/2} = 6.6$; 2: $(U_2^2)^{1/2} = 4.1$					
Expanded Uncertainty (k= 2.3, 10-19 data points)		1: $(6.6 \times 2.3) = 15\%$ 2: $(4.1 \times 2.3) = 9\%$, methandrostenolone = concentration $\pm 15\%$					

Measurement Uncertainty for THG in plasma samples

Symbol	Source of Uncertainty	Value of Units	Distribution	Divisor	Standard Uncertainty	Degree of Freedom (n-1)	Other
U1	Intermediate precision	5.1	N	1	12	13	THG 500 pg/ml
U2	Intermediate precision	4.2	N	1	5.1	13	THG 5000 pg/ml
Combined Uncertainty		1: $(U_1^2)^{1/2} = 12$ 2: $(U_2^2)^{1/2} = 5.1$					
Expanded Uncertainty (k= 2.3, 10-19 data points)		1: $(12 \times 2.3) = 28\%$ 2: $(5.1 \times 2.3) = 12\%$, THG = concentration $\pm 28\%$					

Measurement Uncertainty for trenbolone in plasma samples

Symbol	Source of Uncertainty	Value of Units	Distribution	Divisor	Standard Uncertainty	Degree of Freedom (n-1)	Other
U1	Intermediate precision	7.2	N	1	7.2	13	Trenbolone 500 pg/ml
U2	Intermediate precision	3.8	N	1	3.8	13	Trenbolone 5000 pg/ml
Combined Uncertainty		1: $(U_1^2)^{1/2} = 7.2$; 2: $(U_2^2)^{1/2} = 3.8$					
Expanded Uncertainty (k= 2.3, 10-19 data points)		1: $(7.2 \times 2.3) = 17\%$ 2: $(3.8 \times 2.3) = 9\%$, Trenbolone = concentration $\pm 17\%$					

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Measurement Uncertainty for stanozolol in plasma samples

Symbol	Source of Uncertainty	Value of Units	Distribution	Divisor	Standard Uncertainty	Degree of Freedom (n-1)	Other
U1	Intermediate precision	11	N	1	11	13	Stanozolol 500 pg/ml
U2	Intermediate precision	7.6	N	1	7.6	13	Stanozolol 5000 pg/ml
Combined Uncertainty		1: $(U_1^2)^{1/2} = 11$; 2: $(U_2^2)^{1/2} = 7.6$					
Expanded Uncertainty (k= 2.3, 10-19 data points)		1: $(11 \times 2.3) = 25\%$ 2: $(7.6 \times 2.3) = 17\%$, Stanozolol = concentration $\pm 25\%$					

XIX. POSITIVE SAMPLE DATA PACKET ASSEMBLY ORDER

1. Sample Transfer Sheet (WS # 32)
2. Sample Usage Sheet (Form #7)
3. Confidence Determination Report
4. Sample List
5. Tune Page Settings
6. LC Method
7. MS Method
8. Quantification Report
9. Quantification Calibration Curve
10. MRM Chromatogram Comparison for quantitation including blank plasma, blank solvent immediately before the positive sample, the positive sample, a positive QC sample.
11. MRM Chromatogram Comparison for confirmation including the 3 ion transition traces for the positive sample and a positive QC sample.

Other Required Documentation

In addition to the positive data packet, the following documentation is required:

- Sample list print-out that is maintained in the three-ring binder
- Routine instrument usage checklist completion (and maintenance log if needed)
- Sample Analysis logbook, indicating date, project, operator initials, and listing of official samples and sample identification.

Data packets for samples determined to be negative must contain the following elements:

1. Sample Transfer Sheet (WS # 32)
2. Sample Usage Sheet (Form #7)
3. Confidence Determination Report
4. Quantification Report

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XX. INTERFERING SUBSTANCES

No known substances have been found to interfere with the determination of the anabolic steroids by this procedure.

XX(a). SAFETY REQUIREMENTS

Always wear a lab coat, protective goggles and gloves as the first line of protective measures. All chemicals and reagents **MUST** be opened in a fume hood. Transportation of reagents from the storage room to the workbench **MUST** be accomplished with the use of a reagent rubber bucket. Always observe the “STOP” sign when coming out of the laboratory into the hallway.

XXI. REFERENCES:

1. Fuyu Guan, Cornelius E. Uboh, Lawrence R. Soma, Yi Luo, Jeffery Rudy, and Thomas Tobin. Detection, quantification and confirmation of anabolic steroids in equine plasma by liquid chromatography integrated with tandem mass spectrometry. Rapid Communications in Mass Spectrometry, submitted, September 2004.
2. Fuyu Guan, Cornelius E. Uboh, Lawrence R. Soma, and Yi Luo. Collision-induced decomposition mechanism of anabolic steroids by electrospray ionization tandem mass spectrometry. Rapid Communications in Mass Spectrometry, submitted, September 2004.

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APPENDIX I. FAST SCREENING OF PLASMA SAMPLES FOR THG

The LC mobile phase gradient shown in Table 6 can be adjusted to shorten the analysis time for fast screening purpose. For example, a short LC mobile phase gradient (Table 9) is used for high throughput screening of race horse plasma samples for THG. With the short gradient and the same LC column described earlier in this SOP, the analysis time per sample is only 5 min, half of that from the long LC gradient (Table 6).

To decrease consumption of the limited volume of a race horse plasma sample, sample volume can be reduced from 1.0 mL to 0.5 mL. For 0.5 mL volume of plasma samples, the calibrators and QC samples should be prepared according to Table 10 and Table 11, respectively. The reason for the adjustments in volumes of the working THG solution is that addition of 100 µL of a working THG solution to 0.5 mL of plasma results in undesired milk-like extract in MTBE.

Caution: The short LC gradient (Table 9) is used for screening only. For, quantification and confirmation, the long LC gradient (Table 6) MUST be used.

Table 9. Gradients of LC mobile phase and flow rate for screening for THG

LC Run time (min)	Formate buffer ^a (%)	Methanol (%)	Flow rate (µL/min)
0	23	77	300
2.4	10	90	300
2.5	23	77	300
2.6	23	77	400
4.8	23	77	400
4.9	23	77	300

^a Ammonium formate, 2 mM, pH 3.4.

Table 10. Preparation of Plasma Calibrators for THG

Target Conc. of each anabolic steroid (pg/mL)	Working mixture Solution (ng/mL)	Vol. of Spiked Working Solution (µL)	Volume of Plasma (mL)	Vol. of Spiked 50 ng/mL of IS
25	2.0	12.5	0.50	20
50	2.0	25	0.50	20
100	2.0	50	0.50	20
250	20	12.5	0.50	20
500	20	25	0.50	20
1000	20	50	0.50	20
2500	200	12.5	0.50	20
5000	200	25	0.50	20
10000	200	50	0.50	20

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Table 11. Preparation of Plasma Positive Control (QC) Samples for THG

Target Conc. of each anabolic steroid (pg/mL)	QC Working Solution of the mixture (ng/mL)	Vol. of Spiked QC Working Solution (μL)	Volume of Plasma (mL)	Vol. of Spiked 50 ng/mL of IS
50	2.0	25	0.50	20
50	2.0	25	0.50	20
500	20	25	0.50	20
500	20	25	0.50	20
5000	200	25	0.50	20
5000	200	25	0.50	20

C:\Anabolic\...04_03_04\040304RaceMD002

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Blank pl/0.5 mL, QNP Dec03

RT: 0.00 - 4.01 SM: 5B

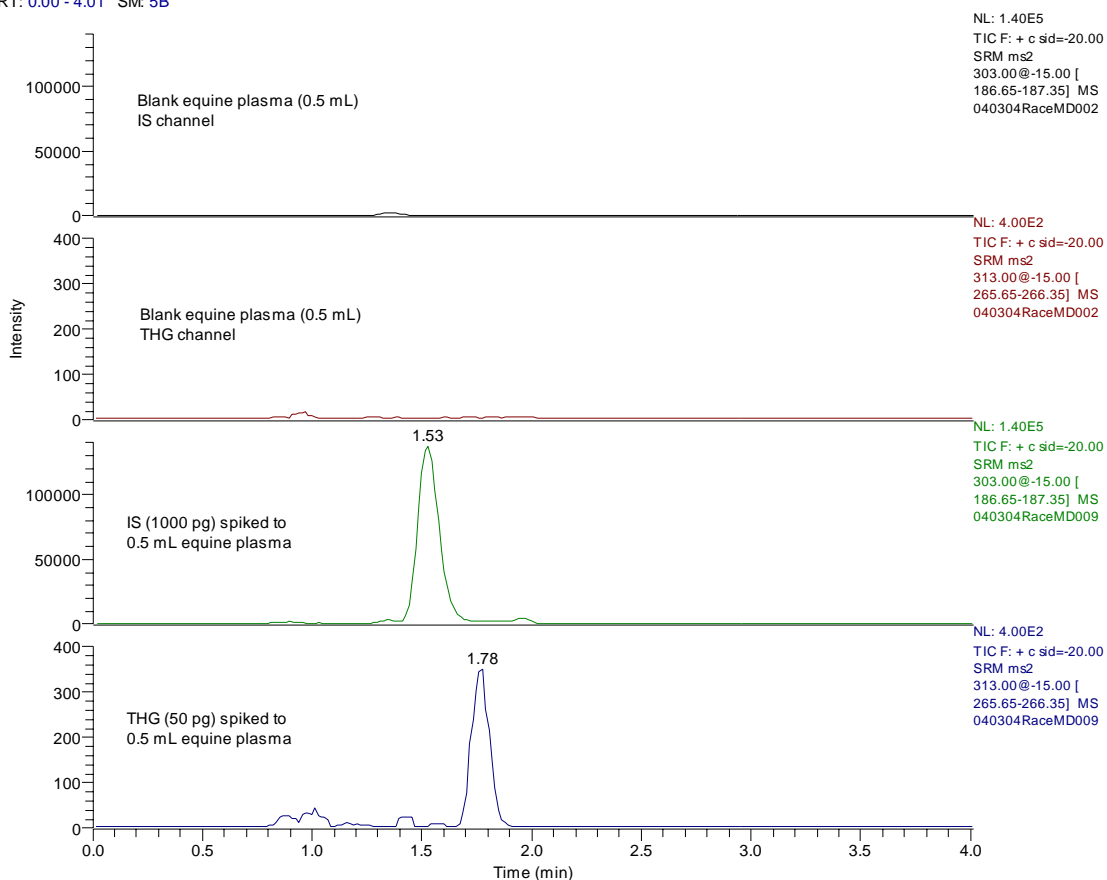


Figure 4. LC-MS/MRM chromatogram of THG at t_R of 1.78 min (the bottom panel) and IS at t_R of 1.53 min (the 2nd panel from bottom) spiked into equine plasma (0.5 mL), and blank equine plasma (top 2 panels) showing detection of THG (50 pg/0.5 mL) under the short LC gradient (Table 9) for fast screening.

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APPENDIX II. PERFORMANCE OF THE METHOD

1. Extraction efficiency is above 74% for the anabolic steroids, as shown in Table 9. Within-run and between-run accuracy is between 80-120%.
2. Matrix effect: ion suppression or enhancement is generally within 10%, as shown in Table 10, and it is insignificant.
3. The anabolic steroids spiked to equine plasma are stable for 24 h at ambient temperature, for 13 days at 4 °C, and for 34 days at -20 °C and -70 °C, as shown in Table 11.

Table 12. Extraction efficiency, accuracy and reproducibility (coefficient of variation, C.V.) for quantification of testosterone, normethandrolone, nandrolone, boldenone, methandrostenolone, THG, trenbolone and stanozolol in equine plasma (n=6) ^a

Added, pg/ml	Extraction recovery (%)							
	Testosterone	Normethandrolone	Nandrolone	Boldenone	Methandrostenolone	THG	Trenbolone	Stanozolol
50	95	105	96	92	92	81	94	82
500	89	86	88	89	86	85	83	74
5000	83	84	84	84	84	80	86	77
	Within-run accuracy							
50	56	57	53	53	54	57	52	50
500	524	532	527	521	518	526	505	569
5000	4654	4826	4712	4654	4648	4663	4745	5561
	Between-run accuracy							
50	48	42	54	45	43	50	46	45
500	503	529	523	510	513	512	519	516
5000	4889	4984	4925	4875	4896	4996	4870	4970
	Within-run C.V. (%)							
50	21	24	19	14	20	19	22	5.8
500	3.3	5.4	5.1	5.0	4.4	7.8	6.3	8.6
5000	4.7	4.3	4.3	4.6	4.4	5.4	4.5	4.4
	Between-run C.V. (%)							
50	20	18	38	11	10	25	10	8.0
500	8.0	5.0	9.0	7.0	7.0	13	8.0	12
5000	4.2	5.6	3.8	4.8	4.7	5.8	4.6	8.4

^a For between run accuracy and precision, six samples were analyzed on three separate days (2 samples each day).

Table 13. Ion suppression or enhancement (%) showing matrix effect for ESI LC-MS/MS of the anabolic steroids ^a

Added pg/mL	Testosterone	Normethandrolone	Nandrolone	Boldenone	Methandrostenolone	THG	Trenbolone	Stanozolol
50	-4.0	8.8	12	0.3	-5.9	-27	-7.4	11
500	-5.8	-9.3	-9.2	-4.9	-8.3	-5.3	-12.3	4.9
5000	-6.1	-7.0	-4.6	-6.0	-6.9	0.6	-7.7	1.8

^a Ion suppression (%) = $[1 - (A_{\text{extr}}/A_{\text{st}})] \times 100$, where A_{st} is the chromatographic peak area of a certain quantity of a drug standard and A_{extr} is the peak area of the same quantity of the drug standard added to the extract of 1.0 mL of equine plasma. Each value is the averaged result of 3 duplicate samples. Negative values mean ion enhancement, and positive values represent ion suppression.

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Table 14. Stability of the anabolic steroids under different storage conditions ^a

Added (pg/mL)	time	Room temperature							
		Testosterone (pg/mL)	Normeth-androlone (pg/mL)	Nandrolone (pg/mL)	Boldenone (pg/mL)	Methandro-sterolone (pg/mL)	THG (pg/mL)	Trenbolone (pg/mL)	Stanozolol (pg/mL)
50	2 h	57	47	61	54	56	51	49	21
50	4 h	50	54	55	54	55	47	57	21
50	6 h	52	61	56	53	55	49	49	32
50	24 h	42	49	44	44	48	37	47	39
500	2 h	530	543	541	538	530	510	504	230
500	4 h	562	577	555	569	566	536	518	303
500	6 h	579	597	588	573	590	530	510	347
500	24 h	532	530	509	535	543	527	505	450
5000	2 h	5210	5165	5143	5176	5112	5026	4670	2284
5000	4 h	5199	5245	5166	5214	5173	4908	4705	2866
5000	6 h	5565	5669	5521	5598	5561	5182	4900	3446
5000	24 h	4955	5018	4637	4952	4951	4768	4538	4029
4 °C									
50	3 days	48	55	52	50	52	45	51	31
50	4 days	61	53	60	38	41	38	46	48
50	12 days	43	48	53	44	51	51	50	46
50	13 days	48	37	51	45	48	42	38	48
500	3 days	552	499	547	547	532	514	507	511
500	4 days	519	477	488	488	484	471	465	516
500	12 days	456	421	450	452	449	461	424	469
500	13 days	463	446	453	463	471	448	457	416
5000	3 days	5148	5010	5251	5251	5045	5092	4972	4822
5000	4 days	4939	4920	5110	5110	4968	4999	4838	5236
5000	12 days	4546	4585	4448	4603	4617	4438	4488	4696
5000	13 days	4734	4663	4738	4735	4735	4094	4523	3658
- 20 °C									
50	13 days	44	46	46	44	44	35	45	55
50	20 days	46	52	50	45	42	46	46	50
50	27 days	47	45	50	52	48	54	46	43
50	34 days	52	47	43	47	49	50	47	50
500	13 days	465	482	475	469	465	451	445	461
500	20 days	475	491	492	477	483	458	464	527
500	27 days	457	462	461	467	466	435	456	453
500	34 days	438	450	425	438	435	418	397	456
5000	13 days	4821	4788	4781	4771	4751	4598	4599	4503
5000	20 days	4687	4723	4638	4674	4656	4581	4469	4729
5000	27 days	4387	4370	4377	4345	4370	4227	4041	4232
5000	34 days	4084	4103	4099	4071	4116	4214	3950	4243

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Table 14. Stability of the anabolic steroids under different storage conditions, continued

Added (pg/mL)	time	Normeth				Methandr		Trenbolo ne (pg/mL)	Stanozolo l (pg/mL)
		Testoster one (pg/mL)	androlon e (pg/mL)	Nandrolo ne (pg/mL)	Boldeno ne (pg/mL)	o- stenolone (pg/mL)	THG (pg/mL)		
-70 °C									
50	13 days	43	46	37	41	42	43	39	47
50	20 days	45	53	69	43	41	45	37	47
50	27 days	49	50	48	47	42	47	46	40
50	34 days	52	50	55	46	51	52	48	47
500	13 days	465	474	453	458	468	467	455	466
500	20 days	486	503	494	468	471	479	466	497
500	27 days	451	479	460	439	436	464	435	428
500	34 days	443	453	452	420	436	444	453	421
5000	13 days	4611	4716	4620	4488	4567	4585	4587	4697
5000	20 days	4478	4514	4379	4192	4387	4310	4135	4650
5000	27 days	4573	4763	4570	4422	4471	4467	4486	4418
5000	34 days	4124	4298	4158	4032	4093	4216	4387	4144

^a Each value was obtained by averaging the results of triple plasma samples (n=3).

APPENDIX III. QUANTIFICATION RESULTS FOR EQUINE PLASMA SAMPLES FROM ADMINISTRATIONS OF THG, BOLDENONE, AND NANDROLONE

Table 15. THG concentration (pg/mL) in plasma and urine samples from THG administration (12 mg per horse of 539 kg body weight, IM)

Time (h)	0	0.25	0.5	0.75	1	2	4	6	8	10	24	48	72
plasma	0	9818	11119	10625	9399	8403	4453	2350	1468	1026	363	40	N/D ^a
EH urine	0						34112	15895	11712	7112			
Neat urine	0						493	228	155	93			

^a Not detected.

THG was detectable 48 h post the single-dose administration and confirmable 24 h post the administration.

Boldenone was detected and quantified in plasma samples up to 29 days post intramuscular administration of a single dose of boldenone undecylenate (1.1 mg/kg, IM), and nandrolone up to 62 days post administration of a single dose of nandrolone decanoate (1.1 mg/kg, IM).

Approved By _____ Laboratory Director

Acknowledged By: _____ Quality Assurance Officer

Acknowledged By: _____ Lab Supervisor

APPENDIX IV. DETECTION, QUANTIFICATION AND CONFIRMATION OF BOLDENONE IN EQUINE PLASMA SAMPLE FROM A HORSE PULL COMPETITION AT A COUNTY FAIR

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09/16/2004 03:11:48 PM

1st Heavy Wt, Kiwanis Wyoming, 9/3/04, 0.5 ml

RT: 0.00 - 5.00 SM: 5B

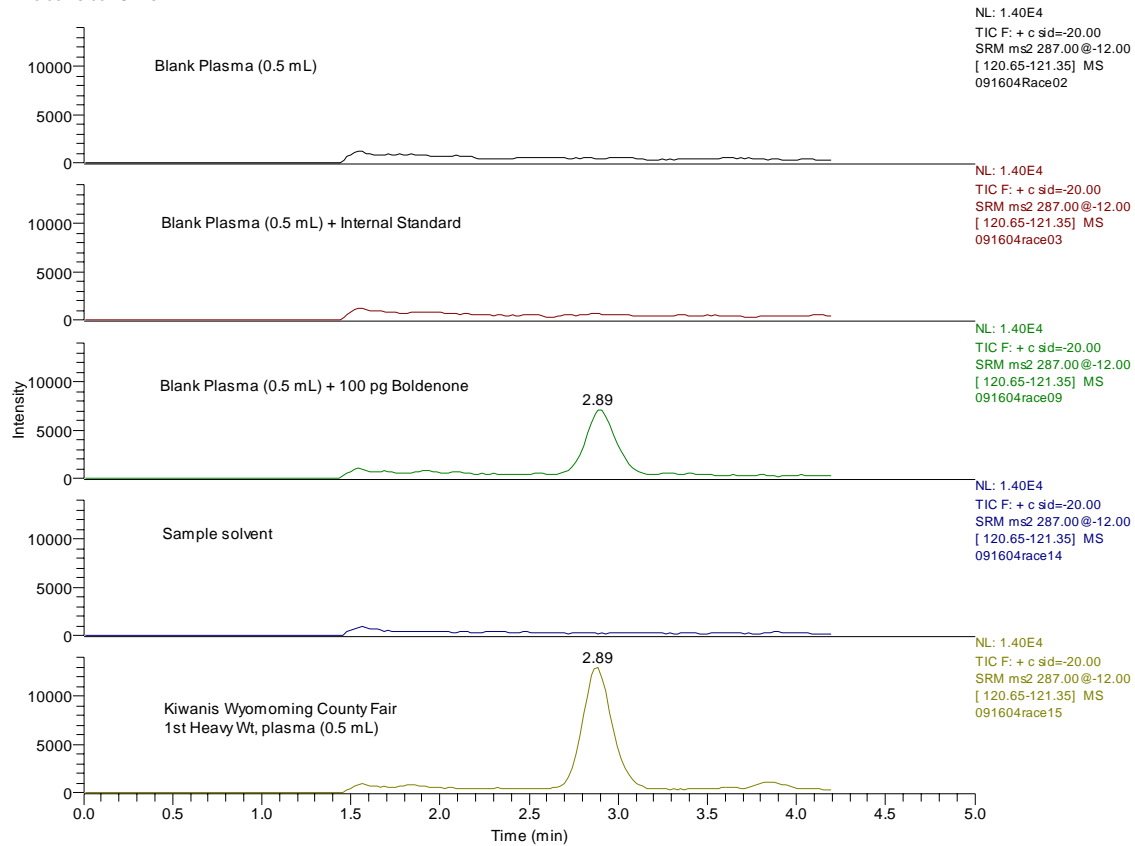


Figure 5. LC-MS/MS chromatograms showing detection of boldenone in an equine plasma sample from a county fair. The 5 chromatograms from top to bottom are from blank plasma (0.50 mL), blank plasma (0.50 mL) plus internal standard, boldenone (100 pg) spiked into the blank plasma (0.50 mL), the sample solvent, and the test plasma sample, respectively.

Approved By _____ Laboratory Director

Acknowledged By: _____ Quality Assurance Officer

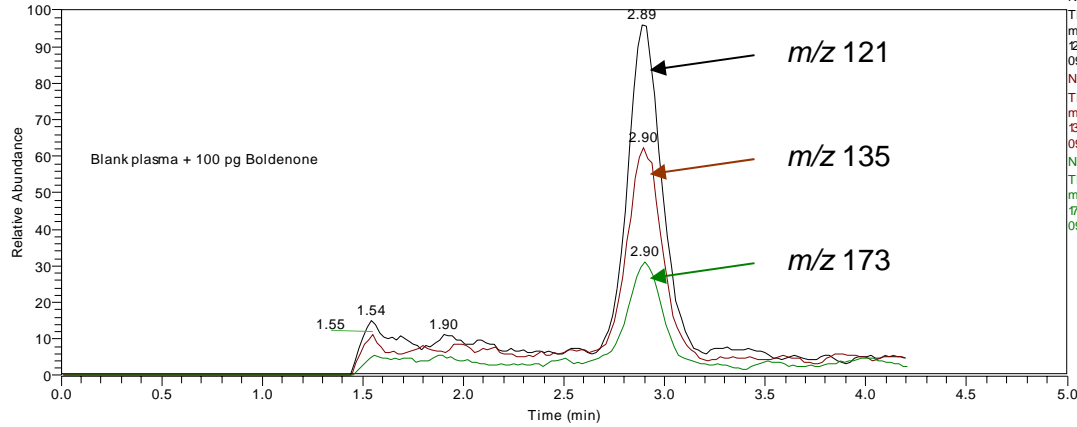
Acknowledged By: _____ Lab Supervisor

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09/16/2004 03:11:48 PM

1st Heavy Wt, Kiwanis Wyoming, 9/3/04, 0.5 ml

RT: 0.00 - 5.00 SM: 5B



RT: 0.00 - 5.00 SM: 5B

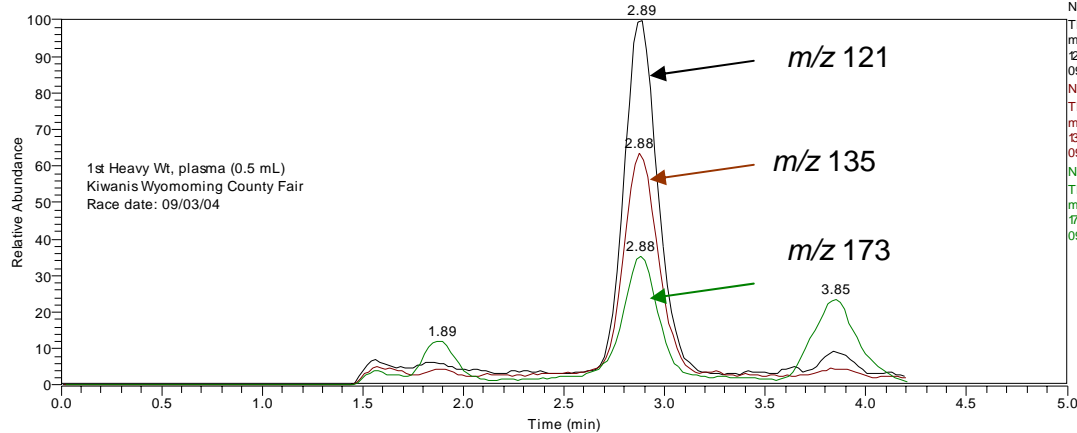


Figure 6. LC-MS/MS chromatograms showing confirmation by product ion ratio of boldenone in an equine plasma sample from a county fair. Top panel: Boldenone (100 pg) spiked into blank plasma (0.50 mL). Bottom panel: confirmation of boldenone in the test sample (0.50 mL).