Standard Operating Procedure

Detection of steroid and non-steroid Hormone in bovine muscle

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1. INTRODUCTION

Steroid hormones are a group of lipophilic, low-molecular weight, and biologically active compounds. Natural and synthetic hormones have been widely used for many decades in animal husbandry to improve the rate of growth and the efficiency of feed conversion. Some hormones might have carcinogenic effect leading to breast cancer, ovarian cancer, prostate cancer and cell carcinoma. Moreover, some synthetic growth promoters have potential endocrine disrupting properties causing behavioral disorders, decreased fertility and birth malformations.

So to ensure consumer's safety, and to detect their presence at very low levels in the food matrices, the development of sensitive, specific and multi-residue analytical methods has become necessary and of a great significance.

2. SCOPE

This standard Operating Procedure (SOP) describes the procedure for screening of growth promoter in animal tissue, using high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

3. OBJECTIVE/PURPOSE

To ensure the accuracy, quality and reproducibility of the procedure / protocol.

4. DEFINITIONS AND ABBREVIATIONS

4.1 HPLC: High-Performance Liquid Chromatography

MS/MS : Tandem mass spectrometry

5. PRINCIPLE OF THE METHOD

Meat sample (5g) was weighed into a 50 ml polypropylene centrifuge tube. An appropriate volume of internal standards was added to each sample to achieve a concentration of 100 µg/kg of progesterone-d9 and 17β-estradiol-d3 and 17β-estradiol-d4. Acetonitrile (10 mL) was added and the tube was shaken for 1 min by vortex. Next, anhydrous NaCl (1g), anhydrous MgSO4 (4g), trisodium citrate

dihydrate (1g) and disodium hydrogenocitrate sesquihydrate (0.5g) were added and the sample was then vortexed again for 1 min. Afterwards, the tube was centrifuged at 3000 rpm for 5 min, and an aliquot of 2 mL of the upper layer was transferred to another centrifuge tube containing anhydrous MgSO4 (300 mg) and Primary Secondary Amine (50 mg). Subsequently, the tube was centrifuged at 3000 rpm for 5 min and the supernatant was transferred to a filtration tube (0.22 µm). Finally, 870 µL of the filtrated supernatant were transferred to an injection vial containing 30 µL of atrazine-d5 (0.5 mg/L) and 100 µL of Lufenuron (1 mg/L).

6. SAFETY CONSIDERATIONS AND PRECAUTIONS

The chemicals use in this procedure can be highly toxic and may constitute a serious health risk for the laboratory workers. Suitable gloves must be worn.

7. MATERIALS AND EQUIPMENT

WATER

Unless stated otherwise, use ultrapure water or deionized water with resistivity of 18.2 $M\Omega.cm^{-1}$

CHEMICALS

7.1.1 Unless stated otherwise, use only the reagents of recognized analytical grade

7.1.2 Ammonium acetate (HPLC grade)

7.1.3 Acetonitrile (CH3CN) (HPLC Grade)

7.1.4 NaCl Sodium Chloride (Analytical grade)

7.1.5 $C_6H_5Na_3O_7.2H_2O$ sodium citrate tribasic dihydrate (Analytical grade)

7.1.6 C6H6Na2O7.1.5H2O sodium hydrogencitrate sesquihydrate (Analytical grade)

7.1.7 $MgSO_4$ (Magnesium Sulfate) (Analytical grade)

7.1.8 PSA (Primary Secondary Amine)

7.2 STANDARD SUBSTANCES

7.2.1 Surrogate standard : progesterone-d9 and of 17β-estradiol d4 , 17β-estradiol d3

7.2.2 internal standard: Atrazin-d5 , lufenuron

7.2.3 Analytical standards: dienestrol, diethylstilbestrol, hexestrol, progesterone, 17α-methyltestosterone, testosterone, melengestrol acetate, 17β-estradiol , estriol, β-estradiol 3 benzoate, 17α-ethinylestradiol, trenbolone acetate and 6 propyl 2 thiouracile.

7.3 EQUIPMENT / APPARATUS

7.3.1 Analytical balance (Range 0.0000g)

7.3.2 Phenex™ Nylon Filter Membranes, 0.20 µm (Phenomemex®)

7.3.3 Laboratory blender (Waring)

7.3.4 Grinder (Moulinex®)

7.3.5 Laboratory centrifuge

7.3.6 Vortex mixer (Velp)

7.3.7 Measuring cylinders (10 mL, 50 mL 100 mL, 250 mL)

7.3.8 Volumetric flask (5 mL, 10 mL, 50 mL)

7.3.9 Stopwatch

7.3.10 Glass beakers

7.3.11 Polypropylene tubes with screw cap (15 mL and 50 mL)

7.3.12 Micropipettes 10–100µL, 100–1000µL, 500–5000µL (Eppendorf)

7.3.13 Amber colour HPLC vials

7.3.14 HPLC-MS/MS system (Agilent 1200 series) consisting of a

7.3.14.1 Quaternary pump(G1311A)

7.3.14.2 Auto sampler (G1313A)

7.3.14.3 Mass spectrometer (Agilent 6410 tandem)

7.3.14.4 Analytical column: Zorbax reversed phase SB-C18 (2.1mm × 100 mm, particle size 3.5 µm)

7.3.14.5 Software: ChemStation for LC 3D (Rev. A. 10.02) software

8. SOLUTIONS

8.1.Mobile phase A

Water (Ammonium Acetate 5mmol/L)

8.2.Mobile phase B

Pure Acetonitrile.

9. PROCEDURE

9.1 TEST PORTION

Grind the meat using the grinder (7.3.4), and weigh 5 ± 0.05 g of the ground sample in 50 ml centrifugation tubes

9.2 NEGATIVE CONTROL

A blank muscle sample was used as control. Weigh 5 ± 0.05 g of ground blank sample in 50 ml centrifugation tubes Continue with the procedure 9.5 and further.

9.3 RECOVERY CONTROL / QUALITY CONTROL SAMPLE

Note: Let fortified samples with standards for at least 45 minutes before continuing with the rest of the procedure.

A blank sample fortified with a known amount of hormone. Weigh 5. ± 0.05 g of ground blank feed sample. Fortify the blank sample to yield 115 ppb concentration of hormones by adding 115 µL of mixture hormones (1ppm) and continue with the procedure 9.5 and further.

9.4 CALIBRATION STANDARDS

Table 1:Preparation of calibration standards

Calibration Standard name	Calibration standard concentration (ppm)	Preparation
CS 1	35	Mix 175 µL of hormones (1000 ppm) with 4825 µL of acetonitrile.
CS 2	1	Mix 57 µL of hormones (CS 1) with 1943 µL of acetonitrile.

9.5 SAMPLE EXTRACTION

Meat sample (5g) was weighed into a 50 ml polypropylene centrifuge tube. An appropriate volume of standards was added to each sample to achieve a concentration of 100 µg/kg of progesterone-d9 and 17β-estradiol-d3 and 17β-estradiol-d4. Acetonitrile (10 mL) was added and the tube was shaken for 1 min by vortex. Next, anhydrous NaCl (1g), anhydrous MgSO4 (4g), trisodium citrate dihydrate (1g) and disodium hydrogenocitrate sesquihydrate (0.5g) were added and the sample was then vortexed again for 1 min. Afterwards, the tube was centrifuged at 3000 rpm for 5 min, and an aliquot of 2 mL of the upper layer was transferred to another centrifuge tube containing anhydrous MgSO4 (300 mg) and Primary Secondary Amine (50 mg). Subsequently, the tube was centrifuged at 3000 rpm for 5 min and the supernatant was transferred to a filtration tube (0.22 µm). Finally, 870 µL of the filtrated supernatant were transferred to an injection vial containing 30 µL of atrazine-d5 (0.5 mg/L) and 100 µL of Lufenuron (1 mg/L).

9.6 METHOD OF ANALYSIS (POSITIF AND NEGATIF HORMONE)

The analysis was carried out on a liquid-phase chromatography (HPLC Agilent Technologies 1200,USA) equipped with a SB-C18 Zorbax reversed phase column,

and coupled to quadrupole triple tandem mass spectrometry via a positive mode and a negative mode electrospray ionization source.

9.7 HPLC-MS/MS ANALYSIS

Flow rate: 0.3 mL/min
Injection volume: 7 µL positive mode / 12 µL negative mode
Total run time: 18 min
Mobile Phase: Water (ammonium acetate 5mmol/L) + acetonitrile
Temperature of column 35°c

9.8 CALCULATION OF HORMONES CONCENTRATION IN A SAMPLE

The concentration of hormones in a sample will be calculated using the calibration curve.

9.9 INTERPRETATION OF TEST RESULTS

HPLC run is considered valid if the following results are achieved.

1. The calibration curve shall give a correlation coefficient (R^2) greater than or equal to 0.99.
2. The recovery controls or quality control samples fortified at 1, 1.5 and 2 µg/kg shall yield recoveries between 60 and 110 %.

If the above two conditions are true, the test samples can be interpreted as follows.

Positive samples:
Samples with corresponding HPLC-MS/MS peak area for above 1 µg/kg

Any other result shall be reported as negative.

10. SCHEMATIC DIAGRAM OF THE EXTRACTION PROCEDURE

Test Sample

1. Grind the meat in blender and weigh 5 ± 0.05 g of ground sample and put in a centrifugation tube of 50 ml

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Sample extraction

1. Fortified the samples at a level of 115µg/kg of surrogate standards.
2. Let the solution 1 hour on the bench before extraction for equilibrium
3. Then add 10 ml of acetonitrile
4. Shak tube on a mechanical shaker for 1 min
5. Add 6.5 g of mixture of salts (4 g $MgSO_4$,1 g NaCl,1g $C_6H_5Na_3O_7,2H_2O$ and 0.5 g $C_6H_6Na_2O_7,1.5H_2O$).
6. Shak the tube on a mechanical shaker for 1 min and centrifuge it at 3000 rpm for 5min.
7. Transfer 2 ml of the supernatant into clean 15 ml centrifuge tube that contain 300 mg $MgSO_4$ + 50 mg PSA
8. The sample was mixed on a mechanical shaker for 1 min in order to purify the extracts.
9. Centrifuge at 3000 rpm for 5 min.
10. Transfer 2 ml to a filtration tube 0.22 µm .
11. Centrifuge at 3000 rpm for 5 min
12. Transfer 870µl of the supernatant into clean injection vial on which was added 30 µl atrazine-d5 at 0.5 ppm and 100µl of lufenuron at 1 ppm like as internals standards,

11. ACCURACY, PRECISION AND UNCERTAINTY CALCULATION

Trueness was determined by calculating recovery from blank samples spiked with at three concentrations ranging 1, 1.5 and 2 ppb . Precision was determined in terms of repeatability (intra-assay precision) and intermediate precision (within-laboratory reproducibility) and was expressed as relative standard deviations (RSD%) of measured analyte .

Analyte	**Spike level (µg/kg)**	**Mean recovery ± SD (%)**	**Repeatability (RSD%)**	**Intermediate precision (RSD%)**
Hormones	1, 1.5, 2	56 - 110	5.6	10.2

12.REFERENCES

1- European Decision 2002/657/EC
2- LC-MS/MS method for the determination of hormones: Validation, application and health risk assessment in various bovine matrices, Fadl Moussa, Samia Mokh, Samah Doumiati, Barbara Barbonic, Nicola Bernabò, Mohamad Al Iskandarani Food and Chemical Toxicology, 2020, DOI: 10.1016/j.fct.2020.111204
3- Development of a new liquid chromatography-tandem mass spectrometry method for the determination of hormones in bovine muscle, Samia Mokh Fadl Moussa, Engie El Koury, Rania Nassar, Nicola Bernabò, Mohamad Al Iskandarani, Journal of Pharmaceutical and Biomedical Analysis, 2020 DOI: 10.1016/j.jpba.2020.113550