United States Department of Agriculture

Food Safety and Inspection Service

CLG-AGON1.11

Screening, Quantitation and Confirmation of Beta-Agonists by UHPLC/MS/MS

This method describes the laboratory procedure for screening, confirmation, and quantitation of beta-agonist residues in bovine and porcine liver and muscle tissues at the minimum levels of applicability listed in Tables 18 and 19.

Notice of Change

There have been several revisions to the method.

A method extension validation was conducted to lower the Minimum Level of Applicability (MLA) for ractopamine from 15 to 3 ppb for porcine and bovine liver and muscle tissue. The MLA for ractopamine in CLG-AGON1 is now consistent with the MLA for CLG-MRM3, which allows for a lower detection limit to screen, confirm, and quantify ractopamine in porcine and bovine liver and muscle tissue. Several technical modifications were also implemented to allow for the lowering of the MLA. These changes include modification of the stock and spiking solution concentration for ractopamine as well as changes to reconstitution solution used for the extraction of ractopamine from liver and muscle tissues.

Table of Contents

Safety Precautions
The personnel performing the analysis are to read the Safety Data Sheets for the standards and reagents used in this method. Follow all applicable federal, state, and local regulations regarding the disposal of chemicals listed in this method.

Introduction

Beta-agonists are a class of veterinarian drugs known as beta-adrenergic agonists[1]. An adrenergic agent is a drug that has effects similar to epinephrine (adrenaline)[1]. Adrenergic drugs either stimulate a response (agonists) or inhibit a response (antagonists). The five categories of adrenergic receptors are: α1, α2, β1, β2, and β3. Agonists vary in specificity between these receptors and may be classified respectively. Thus, beta-agonists stimulate a response of the beta receptors.

Historically beta-agonists were readily administered to livestock animals by the meat production industry as a feed additive[1]. However, studies have shown that beta-agonists can have adverse effects on animal health at high levels with the possibility of carry over effects on human health upon consumption. For example, high levels of beta-agonists have been shown to cause detrimental effects to the central nervous system and cardiovascular system. Some examples of side effects include increased heart rate and blood pressure, anxiety, palpitation, and skeletal muscle tremors.

The Food and Drug Administration (FDA) through the Federal Food, Drug, and Cosmetic Act has the authority to approve and regulate the use of animal drugs. The FDA establishes and publishes regulations setting tolerances for residues of animal drugs. FSIS collects and tests samples for beta-agonists to verify that products meet tolerances and are safe, wholesome, and accurately labeled.

The National Residue Program (NRP) is an interagency program designed to identify, rank, and analyze for residues in meat, poultry, and egg products. FSIS publishes an Annual Sampling Plan to provide information on the process of sampling meat, poultry, and egg products for animal drugs of public health concern. The NRP is monitored and modified annually to set future priorities if data shows trends in detected residues.

Method Overview

The following method describes the laboratory procedure for screening, confirmation, and quantitation of beta-agonist residues (clenbuterol, salbutamol, cimaterol, zilpaterol, and ractopamine) in bovine and porcine liver and muscle tissues.

Free beta-agonist residues are extracted from liver or muscle tissues through a protein-precipitation extraction with a solution containing a mixture of acetonitrile and isopropanol. QuEChERS salts containing sodium chloride, sodium sulfate, and magnesium sulfate are added for further extraction clean up. The final extract is then solvent exchanged and reconstituted with water.

> **Key Definitions**
> **Protein-precipitation:** An extraction technique resulting in solid material being left at the bottom of an extraction vessel with the extract or liquid layer containing the analyte. The liquid layer can be separated out for further analysis.
> **Reversed-phase chromatography:** A type of chromatography that uses a non-polar stationary phase.
> **UHPLC-MS-MS:** An analytical technique where there is a physical separation of target compounds followed by their mass-based detection.

The final extracted residues are examined using reversed-phased chromatography using Ultra-High Performance Liquid Chromatography Coupled with Tandem Mass Spectrometry (UHPLC-MS-MS).

This method may be performed using standards or solutions that contain fewer analytes than the method applicability. When that occurs, the excluded analytes would not be included in the reported results.

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[1] M Smith, "Veterinary Drugs Residues: Anabolics", Encyclopedia of Food Safety 3 (2014) 55-62
[2] DS Hage, JD Carr, "Analytical Chemistry and Quantitative Analysis", Pearson, 2010

Materials and Reagents
- Equipment
- Instrumentation
- Reagents
- Reference Materials

Extraction and Analysis
- Sample Preparation
- AGON Extraction
- Instrumental Analysis

Reporting of Results
- Screening Decision Criteria — Day 3
- Minimum Level of Applicability — Day 3
- Confirmation and Quantitation of Screened Positives — Day 4-5
- Extraction/Analysis — Day 4-5
- Confirmation and Quantitation Decision Criteria — Day 6-7
- Reporting Results — Day 6-7

Days 1-2:
- Solution Preparation
- Standard Preparation
- Sample Preparation
- AGON Extraction
- Instrumental Analysis (up to 16 hours)

Screening Analysis

Business Days 1-2:

Samples Arrive

Extraction/ Analysis

Screening Results Reporting

Business Day 3:

Process Data

Report Results

Result ≥ MLA→ Screen Positive

Results < MLA → Screen Negative

Confirmation and Quantitation Analysis

Business Days 4-5:

Extraction/ Analysis

Business Day 6-7:

Process Data

Report Results

Confirmation

Result ≥ MLA→ Confirm Positive, Violation

Results < MLA →   Residue Not Detected

Quantitation

Tolerance < Result → Residue Detected, Violation

Tolerance > Result ≥ MLA→ Residue Detected, Non-Violative
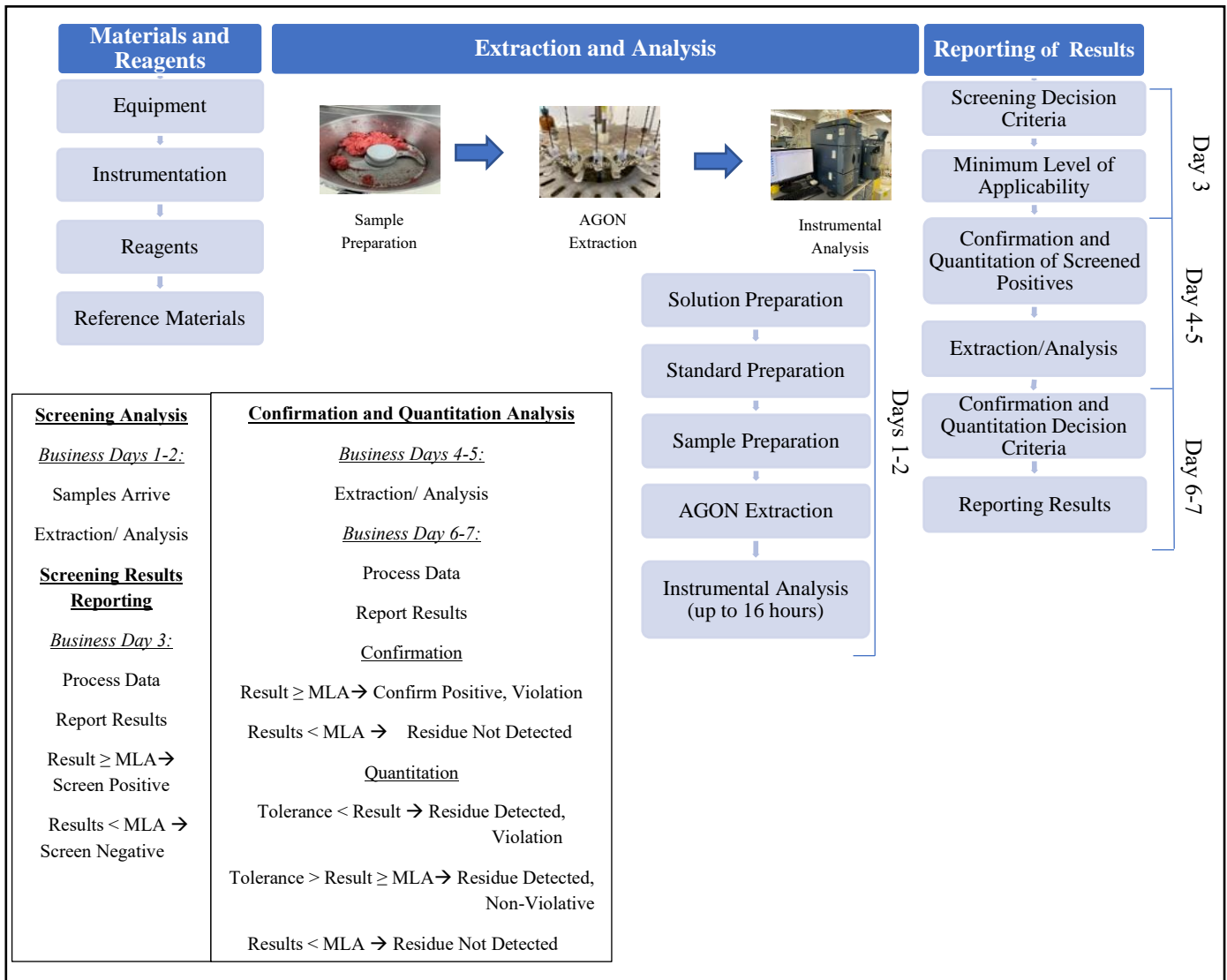
Results < MLA → Residue Not Detected

Figure 1: Overview and timeframe of beta-agonist residue analysis. Materials and reagents are obtained and utilized to prepare solutions and standards. The samples arrive at laboratory, are prepared into a homogenized mixture, weighed, extracted, and analyzed by UHPLC-MS/MS on business days 1-2. Screening results are reported on business day 3. Confirmation and quantitation analysis is done on business days 4-5. Confirmation and quantitation results are reported on business days 6-7. This chart represents the best-case scenario, but analyses may take longer due to analytical testing circumstances. Photos courtesy of Ryan Matsuda, USDA-FSIS.

Decision Criteria

CLG-MRM3 is FSIS's method for screening analytes of several veterinary drugs. If screening results from CLG-MRM3 indicate that beta-agonist residues are found to be "presumptive positive" in porcine and bovine kidney and muscle tissues, CLG-AGON1 is used for confirmation and quantitation analysis.

A sample is considered screened negative by CLG-AGON1 if the results are less than the minimum level of applicability (MLA). The target tissue for beta-agonist residues is liver and therefore if a sample is screened positive, further confirmation or quantitation analyses is to be conducted on the respective bovine and porcine liver samples.

As previously discussed in the introduction chapter of the CLG, confirmation analysis is conducted on residues that have no established tolerances (salbutamol, clenbuterol, and cimaterol). A sample is considered confirmed positive if the results are greater than or equal to the MLA and are confirmed based on the method's decision criteria.

Residues with tolerances (i.e., zilpaterol and ractopamine) undergo confirmation and quantitation analysis. The results are compared to established tolerances and the method's minimum level of applicability (MLA) for zilpaterol and ractopamine for the species and matrix and are confirmed based

> **Key Definitions**
> **MLA:** Lowest level at which an FSIS method has been successfully validated for a residue in each matrix. Full definition is on the CLG website here.

on the method's decision criteria. If the results confirm and are greater than or equal to the MLA but less than the tolerance, the sample is considered detected and non-violative. If the results confirm and are greater than the tolerance, the sample is considered detected and violative.

Disclosure Statement

FSIS does not specifically endorse any test products listed in this method. FSIS acknowledges that equivalent equipment, reagents, or solutions may be suitable for laboratory use. The FSIS laboratory system utilizes the method performance requirements when evaluating the equivalence of an alternative equipment, reagent, or solution for a given analyte and sample matrix pair. Significant equivalence changes would require FSIS laboratory leadership approval.

Materials and Reagents

Equipment

Table 1: Equipment for CLG-AGON1

Equipment	Supplier and Part Number	Purpose
Food Processor	Robot Coupe USA Inc.	Homogenize sample.
Tissuemizer	Polytron	Homogenize sample.
Centrifuge tubes, Polypropylene (PP), 50 mL	General lab supplier	Contain sample material and extraction vessel.
Repeating pipettes and tips, 25 μL, 100 μL, 200 μL, 2.5 mL, 5 mL	General lab supplier	Dispense standards and reagents.
Vortex Mixer	General lab supplier	Facilitates extraction of residue from the sample.

Equipment	Supplier and Part Number	Purpose
Shaker	General lab supplier	Facilitates extraction of residue from the sample.
Centrifuge	General lab supplier	Separates the solid sample material from the extraction solution. Rotor – 50 mL tubes.
Nitrogen Evaporator Apparatus with Heated Water Bath	General lab supplier	Reduces extraction solution down to desired volume.
Whatman Mini-Uniprep syringeless filter vials, 0.2 μm, PVDF	VWR, 12000-524	Filter final extracts.
Analytical Balance	General lab supplier	Record weight of standard reagent. Minimum accuracy ±0.0001g.
Glassware, Class A	General lab supplier	Measuring standards and reagents.

Instrumentation

Table 2: Instrumentation

Instrument	Supplier and Model Number	Purpose
Waters UPLC-MS/MS System	Waters Xevo I-Class LC, Waters Xevo TQD Mass Spectrometer	Extract analysis
Waters UPLC BEH C18, 2.1 × 100 mm, 1.7μm	Waters, 186002352	Extract analysis
Waters VanGuard Pre-column UHPLC BEH C18, 2.1 × 5.0 mm, 1.7 μm	Waters, 186003975	Extract analysis

Reagents

Table 3: Reagents

Reagent	Supplier and Part Number
Acetonitrile (ACN) - LC-MS Grade	General lab supplier
Methanol (MeOH)	General lab supplier
Isopropanol (IPA)	General lab supplier
Water – Resistivity of > 18 MΩ-cm	House system
Formic acid	General lab supplier
Pre-weighed QuEChERS salts (sodium chloride, sodium sulfate, magnesium sulfate)	UCT, ECQUUS8-MP2
Sodium chloride (NaCl), ACS reagent grade	General lab supplier
Sodium sulfate (Na₂SO₄), ACS reagent grade	General lab supplier
Magnesium sulfate (MgSO4)- anhydrous, minimum 99.5% purity	General lab supplier

Reference Materials

Table 4: Reference Materials

Standard	Supplier	Catalog Number
Clenbuterol	USP	1134674
Cimaterol	Millipore Sigma	32568
Salbutamol	USP	1012600
Ractopamine	USP	1598100
Zilpaterol	Millipore Sigma	32379
Ractopamine-d6	Toronto Research Chemicals Inc.	R071402
Zilpaterol-d7	Toronto Research Chemicals Inc.	Z430002

Purity and counterions are to be taken into account when calculating standard concentrations. In-house prepared standards are to be assigned an expiration date that is no later than the stability stated in the method.

Extraction and Analysis

Solution Preparation

Table 5: Preparation of Solutions

Solution	Procedure
Mobile Phase A, 0.1% Formic acid in water	1) Measure 1 mL of formic acid and add to a 1-L volumetric flask. 2) Dilute to volume with water. 3) Mix well and transfer to glass storage container for use.
4:1 Acetonitrile:Isopropanol	1) Using a graduated cylinder measure 800 mL of acetonitrile and add to 1L glass storage container. 2) Using a graduated cylinder measure 200 mL of isopropanol and add to same glass storage container. 3) Mix well for use.
50:50 Methanol:Water	1) Using a graduated cylinder measure 500 mL of methanol and add to 1L glass storage container. 2) Using a graduated cylinder measure 500 mL of water and add to same glass storage container. 3) Mix well for use.

Standard Preparation

Table 6: Stock Standard Solutions

Adjust the concentration whenever using the salt of the analyte of interest to focus on the compound of interest; this includes Salbutamol ● ½H_2SO_4 and Zilpaterol ● HCl. For example, correct the concentration of Clenbuterol ● HCl for salt, since Clenbuterol is the analyte of interest as opposed to Clenbuterol ● HCl. Do not correct the Ractopamine ● HCl concentration for salt since Ractopamine ● HCl is the analyte of interest.

Solution	Procedure
Clenbuterol Stock Standard (~25 µg/mL) Expires after 1 month. Store at 2 - 8 °C.	1) Weigh ~2.5 mg of clenbuterol to the nearest 0.1 mg. 2) Transfer to a 100 mL amber volumetric flask with methanol. 3) Dilute to volume with methanol. 4) Mix well to ensure that clenbuterol is dissolved completely. 5) Transfer to an amber glass storage bottle.

Cimaterol Stock Standard (~25 µg/mL) Expires after 1 month. Store at 2 - 8 °C.	1) Weigh ~2.5 mg of cimaterol to the nearest 0.1 mg. 2) Transfer to a 100 mL amber volumetric flask with acetonitrile. 3) Dilute to volume with acetonitrile. 4) Mix well to ensure that cimaterol is dissolved completely. 5) Transfer to an amber glass storage bottle.
Zilpaterol Stock Standard (~25 µg/mL) Expires after 1 month. Store at 2 - 8 °C.	1) Weigh ~2.5 mg of zilpaterol to the nearest 0.1 mg. 2) Transfer to a 100 mL amber volumetric flask with water. 3) Dilute to volume with water. 4) Mix well to ensure that zilpaterol is dissolved completely. 5) Transfer to an amber glass storage bottle.
Ractopamine Stock Standard (~1000 µg/mL) Expires after 1 month. Store at 2 - 8 °C.	1) Weigh ~10 mg of ractopamine to the nearest 0.1 mg into a 15 mL amber storage container. 2) Add 10 mL of water. 3) Mix through gentle inversion and vortex
Ractopamine-d6 Stock Standard (~100 µg/mL) Expires after 1 year. Store at 2 - 8 °C.	1) Obtain pre-weighed bottle containing 1.0 mg ractopamine-d6. 2) Transfer to a 10 mL amber volumetric flask with 4:1 Acetonitrile:Water. 3) Dilute to volume with 4:1 Acetonitrile:Water. 4) Mix well to ensure that ractopamine-d6 is dissolved completely. 5) Transfer to an amber glass storage bottle.
Zilpaterol-d7 Stock Standard (~20 µg/mL) Expires after 1 month. Store at 2 - 8 °C.	1) Weigh ~1.0 mg of zilpaterol-d7 to the nearest 0.1 mg. 2) Transfer to a 50 mL amber volumetric flask with 4:1 Acetonitrile:Water. 3) Dilute to volume with 4:1 Acetonitrile:Water. 4) Mix well to ensure that zilpaterol-d7 is dissolved completely. 5) Transfer to an amber glass storage bottle.

Salbutamol Stock Standard (~25 µg/mL) Expires after 1 month. Store at 2 - 8 °C.	1) Weigh ~2.5 mg of salbutamol to the nearest 0.1 mg. 2) Transfer to a 100 mL amber volumetric flask with water. 3) Dilute to volume with water. 4) Mix well to ensure to that salbutamol is dissolved completely. 5) Transfer to an amber glass storage bottle.

Table 7: Spiking and intermediate standards

Solution	Procedure
Quantitation spiking standard for ractopamine (5 µg/mL) Expires after 1 month. Store at 2 - 8 °C.	1) Pipette 500 µL of ractopamine stock standard (adjusted for actual stock standard concentration) into a 100 mL amber volumetric flask. 2) Dilute to volume with acetonitrile. 3) Mix well and transfer to an amber glass bottle.
Quantitation spiking standard for ractopamine (0.5 µg/mL) Expires after 1 month. Store at 2 - 8 °C.	1) Pipette 10 mL of the quantitation spiking standard for ractopamine (5 µg/mL) into a 100 mL amber volumetric flask. 2) Dilute to volume with acetonitrile. 3) Mix well and transfer to an amber glass bottle.
Mixed Standard A (250 ng/mL of clenbuterol, 250 ng/mL cimaterol, 250 ng/mL salbutamol, 500 ng/mL zilpaterol, 250 ng/mL ractopamine) If preparing a single standard solution (analysis of single analyte), only add the needed single analyte as opposed to all the compounds. Expires after 1 month. Store at 2 - 8 °C.	1) Pipette ~100 µL of clenbuterol stock standard (adjusted from the determined actual stock standard concentration) into a 10 mL volumetric flask. 2) Pipette ~100 µL of cimaterol stock standard (adjusted from the determined actual stock standard concentration) into the same volumetric flask. 3) Pipette ~100 µL of salbutomol stock standard (adjusted from the determined actual stock standard concentration) into the same volumetric flask. 4) Pipette ~200 µL of zilpaterol stock standard (adjusted from the determined actual stock standard concentration) into the same volumetric flask. 5) Pipette ~500 µL of Quantitation spiking standard for ractopamine (5 µg/mL) into the same volumetric flask. 6) Dilute to volume with water. 7) Mix well and transfer to an amber glass vial.

Solution	Procedure
Mixed standard B (50 ng/mL of clenbuterol, 50 ng/mL cimaterol, 50 ng/mL salbutamol, 100 ng/mL zilpaterol, 50 ng/mL ractopamine) Expires after 1 month. Store at 2 - 8 °C.	1) Add 300 µL of Mixed standard A to a glass vial. 2) Add 1200 µL of water to the same vial. 3) Mix well.
External Standard (3 ng/mL of clenbuterol, 3 ng/mL cimaterol, 3 ng/mL salbutamol, 6 ng/mL zilpaterol, 3 ng/mL ractopamine) Expires after 1 month. Store at 2 - 8 °C.	1) Measure 600 µL of mixed standard B and add to a 10 mL volumetric flask. 2) Dilute to volume with water. 3) Mix well and transfer to an amber glass vial.
Quantitation spiking standard for zilpaterol (0.5 µg/mL) Expires after 1 month. Store at 2 - 8 °C.	1) Pipette 200 µL of the zilpaterol stock standard (adjusted for the actual stock standard concentration) into a 10 mL amber volumetric flask. 2) Dilute to volume with 4:1 Acetonitrile:Isopropanol. 3) Mix well and transfer to an amber glass vial.
Spiking Zilpaterol-d7 Internal standard (2 µg/mL) Expires after 1 month. Store at 2 - 8 °C.	1) Pipette 500 µL of zilpaterol-d7 stock standard into a 5 mL amber volumetric flask. 2) Dilute to volume with 4:1 Acetonitrile:Isopropanol. 3) Mix well and transfer to an amber glass vial.
Ractopamine-d6 Internal standard (5 µg/mL) Expires after 1 month. Store at 2 - 8 °C.	1) Pipette 250 µL of ractopamine-d6 stock standard into a 5 mL amber volumetric flask. 2) Dilute to volume with 4:1 Acetonitrile:Isopropanol. 1) Mix well and transfer to an amber glass vial.

Sample Preparation

Samples must be kept cold before and during shipping to the laboratory. Once received at the laboratory, samples must be frozen (≤ -10°C) prior to grinding if they cannot be prepared on the day of receipt. Once frozen, temper (partially thaw) while keeping it as cold as possible. As shown in Figure 2, trim away fat and connective tissue. As shown in Figure 3, grind tissue in blender or vertical cutter-mixer until homogeneous. Store samples frozen (≤ -10 °C) prior to analysis. Figure 4 shows a frozen homogenized liver sample.

Figure 2: Prepared lean muscle sample with connective tissue removed. Photo courtesy of Hue Quach, USDA FSIS.

Figure 3: Homogenized muscle sample. Photo courtesy of Hue Quach, USDA FSIS

Figure 4: Homogenized frozen liver sample. Photo courtesy of Ryan Matsuda, USDA FSIS

Beta-Agonist Extraction

Samples

Weigh 5 ± 0.1 g of homogenized muscle or liver sample into labeled 50 mL polypropylene centrifuge tubes, as shown in Figure 5.

Figure 5: Weighed controls and samples. Photo courtesy of Ryan Matsuda, USDA-FSIS

QUALITY CONTROL

For screening

1) Weigh three 5 ± 0.1 g portions of blank tissue into 50 mL polypropylene centrifuge tubes. One for the blank (negative control), one for the decision level control, and one for the recovery (positive).

2) Weigh one additional portion for a check sample, if necessary.

For confirmation

1) Weigh three 5 ± 0.1 g portions of blank tissue into 50 mL polypropylene centrifuge tubes. One for the blank (negative control), one for the decision level control, and one for the recovery (positive).

2) Weigh one additional portion for a check sample, if necessary.

For quantitation

1) Weigh two 5 ± 0.1 g portions of blank tissue into 50 mL polypropylene centrifuge tubes. One for the blank (negative control) and one for the recovery (positive).

2) For Zilpaterol, weigh at least six 5.0 ± 0.1 g homogenized liver or muscle tissue portions into 50 mL disposable centrifuge tubes. Additional QCs may be required with large batches.

3) For Ractopamine, weigh 5.0 ± 0.1 g homogenized liver or muscle tissue portions into 50 mL disposable centrifuge tubes, weigh additional tubes according to number for calibration levels and controls listed in Table 8.

Table 8: Controls for ractopamine quantitation

Matrix	Number for blank tissue portions
Porcine Liver	10 Calibration Levels
Porcine Muscle	8 Calibration Levels
Bovine Liver	9 Calibration Levels
Bovine Muscle	7 Calibration Levels

4) Weigh one additional portion for a check sample, if necessary.

> **KEY DEFINITIONS**
> **Negative control (Blank):** A sample that is negative of all analytes.
> **Decision level control:** A sample that is prepared by addition of analytes that have a concentration level comparable to the MLA. Negative and positive controls are compared to "Decision level control".
> **Recovery (positive control):** A sample that is prepared by the addition of analytes that have a concentration level comparable to the MLA. Samples are compared to the recovery.

Analysis

1) Spike controls

    a) For screening and confirmation prepare the decision level and recovery control by fortifying sample with 60 μL of the spiking standard.

    b) For quantitation, follow Tables 9-14.

Table 9: Zilpaterol in Bovine Muscle

Bovine Muscle Cal Level (ng/g)	Zilpaterol (mL)	Spiking Solution (μg/mL)	4:1 ACN:IPA Volume (mL)
0	0.000	0.5	5.000
2	0.020	0.5	4.980
5	0.050	0.5	4.950
10	0.100	0.5	4.900
15	0.150	0.5	4.850
20	0.200	0.5	4.800

Table 10: Zilpaterol in Bovine Liver

Bovine Muscle Cal Level (ng/g)	Zilpaterol (mL)	Spiking Solution (μg/mL)	4:1 ACN:IPA Volume (mL)
0	0.000	0.5	5.000
2.4	0.024	0.5	4.980
6	0.060	0.5	4.940
12	0.120	0.5	4.880
18	0.180	0.5	4.820
24	0.240	0.5	4.760

Table 11: Ractopamine in Bovine Muscle

Bovine Muscle Cal Level (ng/g)	Ractopamine (mL)	Spiking Solution (μg/mL)	4:1 ACN:IPA Volume (mL)
0	0.000	0.5	5.000
3	0.030	0.5	4.970
6	0.060	0.5	4.940
15	0.150	0.5	4.850
30	0.030	5.0	4.970
45	0.045	5.0	4.955
60	0.060	5.0	4.940

Table 12: Ractopamine in Bovine Liver

Bovine Muscle Cal Level (ng/g)	Ractopamine (mL)	Spiking Solution (µg/mL)	4:1 ACN:IPA Volume (mL)
0	0.000	0.5	5.000
3	0.030	0.5	4.970
5	0.050	0.5	4.950
10	0.100	0.5	4.900
18	0.180	0.5	4.820
45	0.045	5.0	4.955
90	0.090	5.0	4.910
135	0.135	5.0	4.865
180	0.180	5.0	4.820

Table 13: Ractopamine in Porcine Muscle

Bovine Muscle Cal Level (ng/g)	Ractopamine (mL)	Spiking Solution (µg/mL)	4:1 ACN:IPA Volume (mL)
0	0.000	0.5	5.000
3	0.030	0.5	4.970
5	0.050	0.5	4.950
10	0.100	0.5	4.900
25	0.025	5.0	4.975
50	0.050	5.0	4.950
75	0.075	5.0	4.925
100	0.100	5.0	4.900

Table 14: Ractopamine in Porcine Liver

Bovine Muscle Cal Level (ng/g)	Ractopamine (mL)	Spiking Solution (µg/mL)	4:1 ACN:IPA Volume (mL)
0	0.000	0.5	5.000
3	0.030	0.5	4.970
5	0.050	0.5	4.950
10	0.100	0.5	4.900
15	0.150	0.5	4.850
30	0.030	5.0	4.970
75	0.075	5.0	4.925
150	0.150	5.0	4.850
225	0.225	5.0	4.775
300	0.300	5.0	4.700

2) Spike internal standard

   a) No internal standard is used for screening and confirmation analysis.

   b) For quantitation, spike the samples with the internal standard for the compound of interest, either 10 µL of Zilpaterol-d7 or 25 µL of Ractopamine-d6.

3) Add 4 mL of acetonitrile and 1 mL of isopropanol.

    a) For screening and confirmation, add to all sample tubes.

    b) For quantitation, only add to the samples and not the controls.

4) For muscle samples, tissuemize for 30 seconds. For liver samples, vortex to mix for 2 minutes.

5) Add 1.2 g NaCl and shake or vortex for 2 minutes.

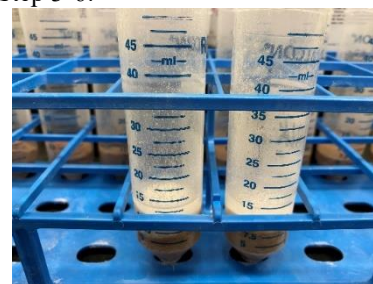6) As shown in Figure 6, add 4 g Na_2SO_4 and 0.5 g $MgSO_4$ and shake or vortex for 2 minutes.

Step 3-6:

Figure 6: QuEChERS salts added for extraction. Photo courtesy of Ryan Matsuda, USDA-FSIS

Optional Stopping Point:

This is a suitable stopping point. Samples may be stored overnight at 2 - 8 °C.

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7) Centrifuge the samples for 5 ± 1 minutes at approximately 2000 RCF at room temperature.

8) Filter extract by:

    a) Whatman mini-uniprep filter vial.

      i. Pipette 0.5 mL of extract into Whatman mini-uniprep filter vial.

      ii. As shown in Figure 7, evaporate to dryness with air or nitrogen.

> Prior to evaporation, each needle in the N-Evap should be cleaned thoroughly through bubbling with acetonitrile for approximately 15 sec. After bubbling, each needle should be carefully wiped dry.

Figure 7: Samples undergoing evaporation. Photo courtesy of Ryan Matsuda, USDA-FSIS

      iii. As shown in Figure 8, add 0.5 mL of 4:1 Acetonitrile:Water to vial, cap with plunger-shaped cap without filtering, vortex for 30 seconds, and filter reconstituted extract by pushing plunger-shaped cap equipped with a 0.2 µm PVDF filter into vial.
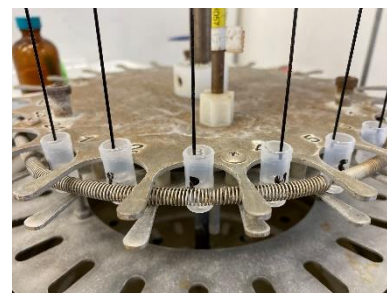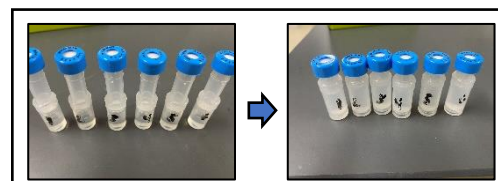
9) Samples are ready to be analyzed by LC-MS/MS

Figure 8: Samples undergoing filtration with mini-uniprep filter vial. Photo courtesy of Ryan Matsuda, USDA-FSIS

Instrumental Analysis

An example of a sample tray for an LC-MS-MS system and an example of an LC-MS-MS instrument are shown in Figure 9 and Figure 10, respectively.

Chromatographic Parameters
1) Mobile phases for AGON analysis

   a) Mobile Phase A – 0.1% formic acid in water

   b) Mobile Phase B – Acetonitrile

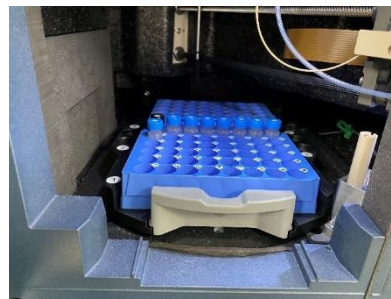2) Flow rate: 0.3 mL/min

3) Run time: 9.50 min

4) Gradient Program

Figure 9: Prepared samples in LC-MS-MS instrument. Photo courtesy of Ryan Matsuda, USDA-FSIS

Table 15: LC Gradient Program

Time (min)	% Mobile Phase A	% Mobile Phase B
0.00	95	5
2.00	95	5
7.50	35	65
8.50	95	5
9.50	95	5

5) Autosampler program

   a) Run time: 9.5 min

   b) Injection Volume: 10 μL

   c) Needle wash solvent: 50:50 Methanol:Water

   d) Sample temperature: 10°C

6) Column manager

   a) Column valve position: To match column location.

   b) Column manager temperature: 50 °C

Instrumental Note:
Autosampler parameters can be modified or optimized if needed to ensure that all chromatographic peaks are present.

Mass Spectrometry Parameters

1) Type: MS/MS

2) Electrospray Source Parameters

   a) Capillary (kV): 3.0

   b) Cone (V): Variable - analyte dependent

   c) Extractor (V): 3.0

   d) RF (V): 0.10

   e) Source Temperature (°C): 150

   f) Desolvation Temperature (°C): 500

   g) Cone Gas Flow (L/hr): 20

   h) Desolvation Gas Flow (L/hr): 1100

   i) Collision Gas Flow (mL/min): 0.20

3) Analyzer Parameters

   a) LM1 Resolution: 10.6

   b) HM 1 Resolution: 14.8

   c) MSMS Mode Entrance: -5

   d) MSMS Mode Collision Energy: Variable – analyte dependent

   e) MSMS Mode Exit: 1

   f) LM 2 Resolution: 8.4

   g) HM 2 Resolution: 12.0

4) MS Method Parameters:

   a) Type: MRM

   b) Ion Mode: ES+

   c) Dwell (s): 0.005

   d) Start time (min): 2.0

   e) End time (min): 5.6

   f) MRM Transitions

> **Instrumental Note:**
>
> Mass spectrometry parameters are optimized and adjusted during annual preventative maintenance and calibration.

Figure 10: LC-MS-MS instrument.
Photo courtesy of Ryan Matsuda, USDA-FSIS

Table 16: MRM Transitions

Analyte	Window (min)	Dwell Times (ms)	Precursor Ion (m/z)	Product Ion (m/z)*	Collision Energy (eV)	Cone (V)
Salbutamol	2.0 – 3.3	0.034	240	**148**	18	18
				166	14	
				121	26	
Cimaterol	2.2 – 3.1	0.034	220	116	50	18
				143	35	
				160	20	
Clenbuterol	4.4 – 5.6	0.052	277	168	35	18
				203	15	
				132	30	
Ractopamine	4.0 – 5.3	0.052	302	107	45	18
				121	26	
				164	18	
Zilpaterol	2.0 – 3.2	0.034	262	157	33	18
				185	25	25
				130	50	18
Ractopamine-D6	4.00 – 5.25	0.034	308	290	12	40
				121	23	
				168	16	
Zilpaterol-D7	2.0 – 3.2	0.052	269	**251**	15	40
				185	21	27
				203	21	27

Most abundant product ion (quant ion) is in bold.

Instrumental Note:

Retention time windows and collision energies were set and utilized at time of method validation.

- Retention time windows may be adjusted to account for aging of UHPLC columns or for improved separation to ensure that all chromatographic peaks are present.
- Collision energies may be adjusted and optimized for improved mass spectrometry detection.
- Target masses for precursor and product ions can be optimized to a *m/z* value that falls within the unit mass resolution of the exact mass, but not to exceed the next integer value (e.g., if the exact mass is 787.5, an allowable target mass range includes 787.0-787.9).

Sample Set

The injection sequence below can be modified, as needed, but must include required controls. System Suitability is to be demonstrated prior to sample set injection.

1) External Standard

2) Calibration Curve (For Quantitation)

3) Decision Level (For Screening and Confirmation)

4) Recovery (Positive Control)

5) Water (Solvent Blank)

6) Blank (Negative Control)

7) Intra-laboratory check sample (if necessary)

8) Water (Solvent Blank; Optional)

9) Samples, up to 24 samples

10) Reinjection of external standard, recovery (positive control), and blank (negative control) if > 24 samples

11) Samples, up to maximum of 48 for set

12) Reinjection of recovery (positive control) or external standard

> **INTRA-LABORATORY**
>
> **CHECK SAMPLE**
>
> Defined on the CLG website here.

Reporting of Results

Decision Criteria

QUALITY CONTROL

Quality Control Procedures

Screening

1) For set acceptance, the analytes in the recovery (positive control) must meet screening criteria.

2) The blank (negative control) must be negative using the screening criteria.

Confirmation

1) For set acceptance, the analytes of interest (i.e. analytes to be confirmed) in the fortified recovery (positive control) must meet confirmation criteria.

2) The blank (negative control) must be negative using the confirmation criteria for the analytes of interest.

Quantitation

1) For set acceptance, the positive control recoveries must be the acceptable recoveries for the tested species listed in Table 17 for all analytes that will be quantitated.

Table 17: Acceptable Recoveries

Tissue	% Recovery
Bovine Muscle	83-118
Bovine Liver	85-109
Porcine Muscle	88-109
Porcine Liver	92-114

2) The blank (negative control) is negative for all analytes that will be quantitated using the quantitation criteria.

Intralaboratory Check Samples (If applicable)

1) Acceptability criteria.
   a) All analytes in the fortified control must meet screening, confirmation, or quantitation criteria.
   b) All analytes in the unfortified control must not meet screening, confirmation, or quantitation criteria.
   c) FSIS Field Service Laboratories are to refer to internal FSIS Quality Control Procedures when unacceptable values are obtained:
      i. Refer to LW-Q1002, Chemistry Non-Conformance Tables, for how to proceed and whether to take corrections or corrective actions.

Screening

1) The screening ion and all product ions for a given analyte must be present. The required ions are listed in Table 16.

2) Each ion must have a signal-to-noise ratio ≥ 3. This may be verified by visual inspection.

3) The retention time of each analyte must match that of the recovery (positive control) or the external standard injected most recently before the sample within 5%.

4) The blank must be negative for all analytes according to the above criteria, with the quant ion peak area being ≤ 10% of the decision level recovery.

5) A water blank injected immediately after the initial positive control injection must be negative for all analytes according to above criteria.

6) The sample is screen positive if the following criteria are met:

   a) The fortified recovery of the analyte must exceed 10% of the decision level recovery.

   b) The sample response equals or exceeds the recovery level.

Confirmation

Confirmation criteria are required only for analytes that are to be quantitated in the sample set.

1) Monitored ions for each analyte will be assessed as follows:
   a) Recovery retention times must match the retention times of the decision level recovery or the external standard within 5%. Retention time for the samples must match the retention time of the recovery (positive control) or the external standard within 5%.

   b) All product ions specified for ratio matching are present with a signal-to-noise ratio ≥ 3. This may be verified by visual inspection.

   c) One of the following ion ratio matching conditions is met:

      i. If two product ions are assessed, one sample ion ratio should match the calculated ratio of the recovery or the external standard within a ±10% absolute difference.

      ii. If three product ions are assessed, the presence of two sample ion ratios should match the ratio of the recovery or the external standard within a ±20% absolute difference.

> **Technical Note:**
>
> Ratios are calculated by dividing the area count of each diagnostic ion by the area count of the base ion. Ion ratios should be less than 1. If the ratio is not less than 1 for a sample set, the inverse of this ratio is to be used.

2) A sample is confirmed positive for an analyte if the above and the following criteria are met:

   a) The recovery (positive control) of the analyte of interest must exceed 10% of the decision level recovery.

   b) The sample response equals or exceeds the appropriate fortified recovery level.

   c) The blank (negative control) must be less than 10% of the decision level recovery.

Quantitation

Quantitation criteria are required only for analytes that are to be quantitated in the sample set.

1) The sample peak retention time must be within ± 5% of a standard (1.5x mid-level calibration standard recommended) or positive control.

2) The quantitative ion must have a signal to noise ratio of ≥ 10.

3) The additional ions for the analyte listed in Table 16 must be present in sample with a signal to noise ratio of ≥ 3.

4) The negative control response must be less than 10% of the positive control run in the same set.

5) The positive control must be within the acceptable recoveries for tested species as listed in Table 17 for the analyte of interest.

6) The determinative coefficient (r^2) for the calibration curve must be ≥ 0.99.

Minimum Level of Applicability

Table 18: Minimum Level of Applicability for Screening and Confirmation level per species

	Bovine Muscle (ppb)	Porcine Muscle (ppb)	Equine Muscle (ppb)	Bovine Liver (ppb)	Porcine Liver (ppb)
clenbuterol	≥ 3	≥ 3	≥ 3	≥ 3	≥ 3
salbutamol	≥ 3	≥ 3	≥ 3	≥ 3	≥ 3
cimaterol	≥ 3	≥ 3	≥ 3	≥ 3	≥ 3
zilpaterol	≥ 6	≥ 6	≥ 6	≥ 6	≥ 6
ractopamine ● HCl	≥ 3	≥ 3	≥ 3	≥ 3	≥ 3

Table 19: Minimum Level of Applicability for Quantitation level per species

	Bovine Muscle (ppb)	Porcine Muscle (ppb)	Equine Muscle (ppb)	Bovine Liver (ppb)	Porcine Liver (ppb)
zilpaterol	≥ 5	N/App	N/App	≥ 6	N/App
ractopamine ● HCl	≥ 3	≥ 3	N/App	≥ 3	≥ 3

N/App = Not applicable

References

21CFR 556 for tolerance values set by FDA.

The National Residue Program sets the number of samples analyzed each year for animal drugs. The National Residue Program Roles Functions and Responsibilities | Food Safety and Inspection Service (usda.gov)

Contact Information and Inquiries

Inquiries about methods can be submitted through the USDA website via the "Ask USDA" portal at https://ask.usda.gov or please contact:

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This method has been validated, reviewed, approved, and deemed suitable and fit for purpose for use in the USDA FSIS Field Service Laboratories.

William K. Shaw, Jr., PhD
Executive Associate for Laboratory Services

Appendix: Example Calculations

Calculations

a) Correction of Analyte Concentration for Salt and Purity

$$\text{Mass}_{\text{Analyte of Interest}} = \text{Mass}_{\text{Analyte Salt}} \times \frac{\text{Molecular Mass}_{\text{Analyte of Interest}}}{\text{Molecular Mass}_{\text{Analyte Salt}}} \times \text{Purity}$$

Where $\text{Mass}_{\text{Analyte Salt}} = {\sim}2.5$ mg

Molecular Mass $_{\text{Analyte of Interest}} = $ _____ g/mol

Molecular Mass $_{\text{Analyte Salt}} = $ _____ g/mol

Purity = ____ (for the product listed)

b) Correction of Clenbuterol Solution Concentration for Salt

$$\text{Conc.}_{\text{Clenbuterol}} = \text{Conc.}_{\text{Clenbuterol} \bullet \text{HCl}} \times \frac{\text{Molecular Mass}_{\text{Clenbuterol}}}{\text{Molecular Mass}_{\text{Clenbuterol} \bullet \text{HCl}}}$$

Where   Concentration $_{\text{Clenbuterol} \bullet \text{HCl}} = 1.000$ mg/mL

Molecular Mass $_{\text{Clenbuterol}} = 277.19$ g/mol

Molecular Mass $_{\text{Clenbuterol} \bullet \text{HCl}} = 313.65$ g/mol

c) Calculation of Actual Concentration from Solid

$$\text{Conc.}_{\text{Analyte of Interest}} = \frac{\text{Mass}_{\text{Analyte of Interest}}}{\text{Volume}_{\text{Solution}}}$$

Where $\text{Mass}_{\text{Analyte of Interest}} = {\sim}1$ or 2.5 mg

Volume $_{\text{Solution}} = 10.0, 50.0,$ or 100.0 mL

d) Calculation of Actual Concentration from Dilution

$$\text{Conc}_{\text{Solution}} = \text{Conc}_{\text{Stock Solution}} \times \frac{\text{Volume}_{\text{Stock Solution}}}{\text{Volume}_{\text{Solution}}}$$

e) Adjustment of Volume Pipetted for Actual Concentration

$$\text{Volume}_{\text{actual}} = \text{Volume}_{\text{approximate}} \times \frac{\text{Conc}_{\text{approximate}}}{\text{Conc}_{\text{actual}}}$$

Estimated Amount Found

This is a quantitative estimate calculated for comparison to the screen cutoff level. It is based on a one point calibration with the positive control injected most recently before the sample as the reference. The MS instruments can be programmed to automatically do this calculation.

$$D = E * B \text{ sample} / B \text{ pos. ctrl.}$$

Where   D = Estimated Amount Found in the Sample (ppb)

E = Positive Control Fortification Level (ppb)

B sample = Quant Ion Peak Area in the Sample (counts)

B pos. ctrl. = Quant Ion Peak Area in the Positive Control injected most recently before the sample (counts)

Quantitation Calculation

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

b) Calculate the regression parameters for the calibration curve using the linear regression formula,

y= mx + b

where:

y= area ratio of the analyte/internal standard

x= concentration of standards (ppb)

c) The coefficient of correlation (r^2) must be $\geq$ 0.99.

d) Do not use the origin as a data regression point.

e) Determine sample concentrations using the linear regression formula.