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Title: Screening, Determination and Confirmation of Beta- Agonists by LC/MS/MS		
Revision: 09	Replaces: CLG- AGON1.08	Effective: 05/18/2018

Contents

A. INTRODUCTION

1. Background

Beta-agonists are also known as beta-adrenergic agonists. An adrenergic agent is a drug that has effects similar to epinephrine (adrenaline). 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, and agonists vary in specificity between these receptors, and may be classified respectively. Thus, beta-agonists stimulate a response of the beta receptors.

Beta-agonists are used for growth promotion in food animals, increasing lean muscle mass. In humans, clenbuterol and salbutamol are used as bronchodilators by asthma sufferers and as performance-enhancing drugs by athletes. Human side effects include increased heart rate and blood pressure, anxiety, palpitation, and skeletal muscle tremors.

2. Summary of Procedure

Free residues of clenbuterol, salbutamol, cimaterol, zilpaterol, and ractopamine are extracted from liver or muscle tissues with a mixture of acetonitrile and isopropanol. Sodium chloride, sodium sulfate, and magnesium sulfate salts are used to precipitate proteins and dehydrate the solution. This extract is evaporated, reconstituted in water, filtered, and analyzed by LC/MS/MS.

3. Applicability

This method is suitable for the screening, confirmation and/or quantitation of β-agonists at the levels listed in Tables 1 and 2.

Table 1 –Screening and Confirmation levels

	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	≥ 15	≥ 15	≥ 15	≥ 15	≥ 15

Table 2 –Quantitation levels

	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	≥ 15	≥ 25	N/App	≥ 45	≥ 75

Note: Refer to 21CFR for tolerance values set by FDA and 40CFR for tolerance values set by EPA.

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus
   a. Waring commercial blender Model 51BL31.
   b. Tissuemizer – Polytron, Kinematica AG, Model No. PT2100.
   c. Top loading balance – 0.01 g sensitivity, PJ3600 DeltaRange, Mettler.
   d. Centrifuge tubes – 50 mL round bottom polyallomer with sealing caps, Cat. No. 3138-0050, Nalgene.
   e. Centrifuge tubes – 50 mL conical, disposable, polypropylene, with caps, Cat. No. 352098, Becton Dickinson
   f. Micropipettes – Adjustable, 10 – 5000 µL, Eppendorf.
   g. Vortex mixer – variable speed, Cat. No. S8223-1, American Scientific Products.
   h. Shaker – Horizontal flatbed, two speed, Cat. No. 511105, Eberbach.
   i. Centrifuge – International Equipment Company B-22M high speed with rotor 876 for 50 mL tubes, Cat. No. 20671-007, VWR Scientific.
   j. Syringeless filter device – Mini-UniPrep, 0.2µm nylon, Cat. No. UN203NPUNYL, Whatman
   k. Evaporator – N-Evap 112, Organomation Associates Inc., Model No. 8125.
   l. Filter – 0.2 µm, nylon, acrodisc-13, Cat. No. 4551, Pall Gelman Sciences, Inc.
   m. Amber autosampler vial, 12 x 32 mm, E&K, Cat. No. E251011
   n. Analytical balance – 0.0001 g sensitivity, AG204, Mettler.
   o. Volumetric flasks –100 mL amber, 10 mL amber.
   p. Graduated cylinder– 1L, Class A.

q. Food processor – Robot Coupe model RSI6Y-1, Robot Coupe USA Inc.

r. Sample cups – eValue 4.5 oz specimen containers with caps, Cat. No. C686550, E&K Scientific.

2. Instrumentation

a. Waters Acquity I-Class with Waters Xevo TQD mass spectrometer.

b. Guard column: Acquity UPLC BEH C18 VanGuard Pre-Column, 2.1 x 5 mm, 1.7 µm particles, Cat. No. 186003975.

c. UPLC Column: Acquity UPLC BEH C18, 1.7 µm, 2.1 mm x 100 mm, Cat. No. 186002352.

C. REAGENTS AND SOLUTIONS

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

1. Reagents

a. Methanol (MeOH) – HPLC grade, Cat. No. 230-4, Burdick & Jackson

b. Acetonitrile (ACN) – HPLC grade, Cat. No. 015-4, Burdick & Jackson.

c. Isopropanol (IPA) – HPLC grade, Cat. No. AH323-4, Burdick & Jackson.

d. Sodium chloride (NaCl) – ACS reagent grade, Cat. No. S271-1, Fisher.

e. Sodium sulfate – ACS reagent grade, Cat. No. 354250010, Acros Organics.

f. Magnesium sulfate - anhydrous, minimum 99.5% purity, Cat. No. M-7506, Sigma.

g. Pre-weighed salts (NaCl; $MgSO_4$ and Na_2SO_4) – Cat. No. ECQUUS8-MP2, UCT can be used instead of d,e and f above

h. Water – Deionized, HPLC grade, Millipore Rx system.

i. Formic acid – Purity 98 - 100%, Cat. No. 27001, Riedel-de Haën.

2. Solutions

a. Aqueous mobile phase (0.1% formic acid in water):

Pipette 1 mL of formic acid into a 1L class A graduated cylinder. Fill to volume with Millipore water.

b. 4:1 Acetonitrile/Isopropanol Solution

Add 800 mL Acetonitrile and 200 mL Isopropanol into a 1L class A graduated cylinder.

D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counter ions are to be taken into account when calculating standard concentrations. In-house prepared standards shall be assigned an expiration date that is no later than the stability stated in the method. The maximum length of time that an in-house prepared standard shall be used is 1 year unless the laboratory has produced extension data.

1. Standard Information

   a. Clenbuterol (CLEN) ● HCl– 95% pure, Cat. No. C5423, Sigma Chemical Co.

   b. Cimaterol (CIM) – approximately 99% pure, Cat. No. 0435/50, Tocris Bioscience.

   c. Salbutamol (SAL) ● ½H_2SO_4 – approximately 99% pure, Cat. No. 0634/250, Tocris Bioscience.

   d. Ractopamine ● HCl (RAC) – Elanco Animal Health.

   e. Ractopamine-d6-HCl (RAC-d6) – Cat. No. R071402, Toronto Research Chemicals Inc.

   f. Zilpaterol ● HCl (ZIL) – Merck Co.

   g. Zilpaterol- d7 (ZIL-d7) – Cat. No. Z430002, Toronto Research Chemicals Inc.

2. Preparation of Standard Solution(s)

   Note: Adjust all standard weights for purity. The concentration of Clenbuterol ● HCl is to be corrected for salt, since Clenbuterol is the analyte of interest as opposed to Clenbuterol ● HCl. The Ractopamine ● HCl concentration is not to be corrected for salt since Ractopamine ● HCl is the analyte of interest. Adjust concentration whenever using the salt of the analyte of interest instead of the ion of interest; this includes Salbutamol ● ½H_2SO_4 and Zilpaterol ● HCl. See calculations in section G.

   a. CLEN, CIM, SAL and ZIL stock standards (~25 µg/mL):

      Weigh ~2.5 mg of CLEN, CIM, SAL and ZIL into its own 100 mL amber volumetric flask and bring to volume with the appropriate solvent: for CLEN, methanol; for CIM, acetonitrile; for SAL & ZIL, Millipore water. Record the weight to 0.1 mg and calculate the exact concentration. These standards are stable for 1 month when stored in a refrigerator at 2 - 8 °C.

   b. RAC stock standard (~100 µg /mL):

      Weigh ~10.0 mg into a 100 mL amber volumetric flask and dilute to volume with Millipore water. Record the weight to 0.1 mg and calculate the exact concentration. This standard is stable for 1 month when stored in a refrigerator at 2 - 8 °C.

   c. Mixed intermediate standard A (250 ng/mL CLEN, CIM, SAL, 1250 ng/mL RAC; 500 ng/mL ZIL):

Pipette ~100 µL (adjusted for the actual stock standard concentration) of the CLEN, CIM, SAL stock standards, ~200 µL of the ZIL stock standard, and ~125 µL of the 100 µg/mL RAC stock standard into a 10 mL amber volumetric flask and bring to volume with Millipore water. This standard is stable for 1 month when stored in a refrigerator at 2 - 8 °C.

d. Mixed intermediate standard B (50 ng/mL CLEN, CIM, SAL, 250 ng/mL RAC; 100 ng/mL ZIL):

Pipette 200 µL of the mixed intermediate standard A into an HPLC vial and add 800 µL Millipore water. This standard is stable for 1 month when stored in a refrigerator at 2 - 8 °C.

e. Mixed external standard (3 ng/mL CLEN, CIM, SAL, 15 ng/ mL RAC; 6 ng/mL ZIL):

Pipette 60 µL of mixed intermediate standard B and 940 µL of Millipore water into an HPLC vial. This standard is stable for 1 month when stored in a refrigerator at 2 - 8 °C.

f. Quantitation spiking standard for ractopamine (2.5 µg/mL):

Pipette 250 µL of RAC stock standard (adjusted for the actual stock standard concentration) into a 10 mL amber volumetric flask and bring to volume with a 4:1 acetonitrile / Isopropanol solution. This standard is stable for 1 month when stored in a refrigerator at 2 – 8 °C. For calibration curve preparation, refer to table 3 in section F.2 for detail.

g. Quantitation spiking standard for zilpaterol (0.5 µg/mL):

Pipette 200 µL of ZIL stock standard (adjusted for the actual stock standard concentration) into a 10 mL amber volumetric flask and bring to volume with a 4:1 acetonitrile/isopropanol solution. This standard is stable for 1 month when stored in a refrigerator at 2-8°C. For calibration curve preparation, refer to table 3 in section F.2 for details

h. ZIL-d7 stock standard (~20 µg/mL) :

Weigh ~1 mg of ZIL into a 50 mL amber volumetric flask and bring to volume with a 4:1 Acetonitrile / Isopropanol Solution. Record the weight to 0.1 mg and calculate the exact concentration. This standard is stable for 1 month when stored in a refrigerator at 2-8 °C.

i. RAC-d6 stock standard (100 µg/mL):

Weigh 1 mg of RAC-D6 into a 10 mL volumetric flask and bring to volume with a 4:1 Acetonitrile / Isopropanol Solution. This standard is stable for 1 year when stored in a refrigerator 2-8 °C.

j. ZIL-d7 Internal standard (2 µg/mL):

Pipette 500 µL of ZIL-D7 stock standard into a 5 mL amber volumetric flask and

bring to volume with volume with a 4:1 acetonitrile/isopropanol solution. This standard is stable for 1 month when stored in a refrigerator at 2-8 °C.

k. RAC-d6 Internal standard (5 µg/mL):

Pipette 250 µL of RAC-D6 stock standard into a 5 mL amber volumetric flask and bring to volume with volume with a 4:1 acetonitrile/isopropanol solution. This standard is stable for 1 month when stored in a refrigerator at 2-8 °C.

E. SAMPLE PREPARATION

1. Liver and Muscle homogenization

   a. Blender or food processor

      i. Cut liver or muscle sample into smaller pieces and homogenize in a blender or food processor.

      ii. Transfer homogenized sample into a plastic bag and store in a freezer at ≤ -10 ºC.

      iii. Let sample partially thaw prior to analysis.

   b. Alternatively, dry ice grinding can be used for muscle sample homogenization

      i. Chop 0.5 - 1 lb of muscle tissue into small pieces and homogenize with an equal amount of dry ice in a large food processor. The resulting sample homogenate will be a frozen powder.

      ii. Transfer a portion of the homogenized sample into a loosely capped sample cup until the dry ice has sublimed. Excess sample from step E.1.b.i. may be discarded.

      iii. Tighten the caps and store in a freezer at ≤ -10 ºC .

F. ANALYTICAL PROCEDURE

1. Preparation of Controls for Liver and Muscle Tissue for Screening and Confirmation.

   a. Weigh 5.0 ± 0.1 g blank homogenized tissue portions into 50 mL disposable centrifuge tubes.

   b. Screening - Prepare one of each for a decision level recovery, a recovery (positive control), blank (negative control) and a check sample if necessary. Prepare recoveries by fortifying with 60 µL of the mixed intermediate standard A (D.2.c.).

   c. Confirmation – Prepare one each for a decision level recovery, a blank (negative control) and a recovery (positive control). Prepare recoveries by fortifying with 60 µL of the mixed intermediate standard A (D.2.c).

2. Quantitation - Preparation of Calibration curve and Controls for Liver and Muscle tissue.

   a. Weigh at least six 5.0 ± 0.1 g blank homogenized liver or muscle tissue portions into 50 mL disposable centrifuge tubes. Additional QCs may be required with large batches.

   b. Referencing Table 3, prepare a calibration curve, recovery (positive control), and blank (negative control):

      i. Fortify at least 4 samples with the compound of interest and its internal standard at non-zero levels that bracket the targeted concentration.

      ii. Fortify a recovery (positive control) with the compound of interest and its internal standard at 0.5X.

      iii. Fortify a blank (negative control) with the internal standard for the compound of interest at 0X.

   c. Referencing Table 5 bring the total volume for the calibration curve, recovery (positive control), and blank (negative control) to 5 mL with the specified amount of 4:1 Acetonitrile / Isopropanol solution.

Table 3 Calibration Curve

	Ractopamine (µL)				**Zilpaterol (µL)**		**Zilpaterol-d7 (IS) (µL)**	**Ractopamine-d6 (IS) (µL)**
Spiking levels	**Bovine**		**Porcine**		**Bovine**			
	Muscle	**Liver**	**Muscle**	**Liver**	**Muscle**	**Liver**		
0X	0	0	0	0	0	0	10	25
0.2X	12.0	36.0	20.0	60.0	20.0	24.0	10	25
0.5X	30.0	90.0	50.0	150.0	50.0	60.0	10	25
X	60.0	180.0	100.0	300.0	100.0	120.0	10	25
1.5X	90.0	270.0	150.0	450.0	150.0	180.0	10	25
2X	120.0	360.0	200.0	600.0	200.0	240.0	10	25

Table 4 – Calibration Curve Concentrations (ppb)

	Ractopamine				Zilpaterol	
Spiking Levels	Bovine		Porcine		Bovine	
	Muscle	Liver	Muscle	Liver	Muscle	Liver
0X	0	0	0	0	0	0
0.2X	6	18	10	30	2	2.4
0.5X	15	45	25	75	5	6
1X	30	90	50	150	10	12
1.5X	45	135	75	225	15	18
2X	60	180	100	300	20	24

Table 5 – Additional dilution volume for quantitation standards

		Ractopamine				**Zilpaterol**	
Spiking levels	**4:1 ACN/IPA**	**Bovine**		**Porcine**		**Bovine**	
		Muscle	**Liver**	**Muscle**	**Liver**	**Muscle**	**Liver**
0X	Volume (mL)	5.00	5.00	5.00	5.00	5.00	5.00
0.2X	Volume (mL)	4.99	4.96	4.97	4.96	4.98	4.98
0.5X	Volume (mL)	4.97	4.91	4.92	4.90	4.95	4.94
X	Volume (mL)	4.94	4.82	4.83	4.80	4.90	4.88
1.5X	Volume (mL)	4.91	4.73	4.75	4.70	4.85	4.82
2X	Volume (mL)	4.88	4.64	4.67	4.60	4.80	4.76

3. Extraction Sample Extraction for Liver and Muscle Tissue

   a. Weigh 5.0 ± 0.1 g homogenized tissue into a 50 mL disposable centrifuge tube. Include the prepared recoveries and blank (negative control) in the sample set at this time. **For quantitation only: spike the samples with the internal standard for the compound of interest, either 10 µL of Zilpaterol-d7 or 25 µL of Ractopamine-d6.**

   b. Add 4 mL acetonitrile and 1 mL isopropanol to all sample tubes for the screening and confirmation sets. **For quantitation set: only add these to samples and not the controls.**

c. For dry ice ground muscle (E.1.b.), cap and shake or vortex for 2 minutes. For muscle homogenized without dry ice (E.1.a), tissuemize for 30 seconds.

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

e. Add 4 g Na_2SO_4 and 0.5 g $MgSO_4$ and shake or vortex for 2 minutes.

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

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

g. Filter extract by either of:

i. Whatman mini-uniprep filter vial.

(a) Pipette 0.5 mL of extract into Whatman mini-uniprep filter vial.

(b) Evaporate to dryness with air or nitrogen.

(c) Add 0.5 mL Millipore 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 0.2µm nylon filter into vial.

ii. 3 mL syringe and 0.2µm nylon filter.

(a) Pipette 0.8 mL of extract into a 12x75 mm glass tube.

(b) Evaporate to dryness with air or nitrogen.

(c) Add 0.8 mL of Millipore water to the glass tube, vortex for 30 seconds, and filter using a 3 mL syringe and 0.2µm nylon filter into a vial.

4. Instrumental Settings

Note: The instrument parameters may be optimized to ensure system suitability.

a. UPLC Conditions:

Aqueous Mobile Phase	0.1% formic acid in water
Organic Mobile Phase	Acetonitrile
Flow Rate	0.3 mL/min
Column Temperature	50 °C
Injection Volume	10 µL
Run Time	9.5 minutes

b. UPLC Mobile Phase Gradient Table:

Time	% Aqueous	% Organic
0.00	95%	5%
2.00	95%	5%
7.50	35%	65%
8.50	95%	5%
9.50	95%	5%

c. MS Parameters:

i. Type: MS/MS

ii. Electrospray Source Parameters:

(a) Capillary (kV): 3.00

(b) Cone (V): 40

(c) Desolvation Temperature (°C): 500

(d) Desolvation Gas Flow (L/Hr): 1100

(e) Cone Gas Flow (L/Hr): 20

iii. Analyzer Parameters:

(a) LM Resolution 1: 10.6

(b) HM Resolution 1: 14.8

(c) Ion Energy 1: -0.3

(d) LM Resolution 2: 8.4

(e) HM Resolution 2: 12.0

(f) Ion Energy 2: 0.1

d. Collision Energy: variable, see MRM parameters

e. MRM Parameters:

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** 166 121	18 14 26	18
Cimaterol	2.2 – 3.1	0.034	220	116 143 **160**	50 35 20	18
Clenbuterol	4.4 – 5.6	0.052	277	168 **203** 132	35 15 30	18
Ractopamine	4.0 – 5.3	0.052	302	107 121 **164**	45 26 18	18
Zilpaterol	2.0 – 3.2	0.034	262	157 **185** 130	33 25 50	18 25 18
Ractopamine-D6	4.00 – 5.25	0.034	308	290 121 **168**	12 23 16	40
Zilpaterol-D7	2.0 – 3.2	0.052	269	**251** 185 203	15 21 21	40 27 27

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

5. Injection sequence (if applicable)/Sample Set
   a. External Standard or Calibration Curve
   b. Decision Level Recovery
   c. Recovery (Positive Control)
   d. Water Blank
   e. Blank (Negative Control)
   f. Intra-laboratory check sample (if needed)
   g. Water Blank (optional)
   h. Samples, up to 24
   i. Reinjection of external standard, recovery (positive control), and blank (negative control) if > 24 samples
   j. Samples, up to maximum of 48 for set

k. Reinjection of recovery (positive control) (for system suitability)

Note: A new set including all QC's will be started after 48 samples.

G. CALCULATIONS / IDENTIFICATION

1. 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 $\text{Mass}_{\text{Analyte Salt}}$ = ~2.5 mg

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

$\text{Molecular Mass}_{\text{Analyte Salt}}$ = _____ 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 $\text{Concentration}_{\text{Clenbuterol} \bullet \text{HCl}}$ = 1.000 mg/mL

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

$\text{Molecular Mass}_{\text{Clenbuterol} \bullet \text{HCl}}$ = 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 $\text{Mass}_{\text{Analyte of Interest}}$ = ~1 or 2.5 mg

$\text{Volume}_{\text{Solution}}$ = 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

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

2. 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 sample / B 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)

3. 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 ≥ 0.99.
   d. Do not use the origin as a data regression point.
   e. Determine sample concentrations using the linear regression formula.

4. Screening Criteria
   a. The screening ion and all product ions for a given analyte must be present. The required ions are listed in F.4.e.
   b. Each ion must have a signal-to-noise ratio ≥ 3. This may be verified by visual inspection.
   c. 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%.
   d. The blank must be negative for all analytes according to the criteria (G.4.a-c) above, with the quant ion peak area being ≤ 10% of the decision level recovery.
   e. A water blank injected immediately after the initial positive control injection must

be negative for all analytes according to criteria G.4.a.-c. above.

f. The sample is screen positive if the following criteria are met:

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

ii. The sample response equals or exceeds the recovery level.

5. Confirmation Criteria

a. Monitored ions for each analyte will be assessed as follows:

i. 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%.

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

iii. One of the following ion ratio matching conditions is met:

(a) 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.

(b) 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.

Note: Ratios are calculated by dividing the area count of each diagnostic ion by the amount 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 may be used.

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

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

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

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

6. Quantitation Criteria

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

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

c. The additional ions for the analyte listed in F.4.e. must be present in sample with a signal to noise ratio of ≥ 3.

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

e. The positive control must be 60-110% for the analyte of interest .

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

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

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment — safety glasses and/or face shield, disposable gloves, lab coat.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Methanol, acetonitrile, and isopropanol	Flammable and poisonous	Use reagents in an efficient fume hood away from all electrical devices and open flames. Wear gloves and protective eyewear.
Formic acid	Acid burns	Wear protective equipment and avoid contact with skin.

3. Disposal Procedures

Follow federal, state and local regulations

I. QUALITY ASSURANCE PLAN

1. Performance Standard

a. For screening:

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

ii. The blank (negative control) must be negative using the criteria in Section G.

b. For confirmation:

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

ii. The blank (negative control) must be negative using the criteria in Section G for the analytes of interest.

c. For quantitation:

i. For set acceptance, the positive control recoveries must be 60-110% for all analytes that will be quantitated.

ii. The blank (negative control) is negative for all analytes that will be quantitated using the criteria in Section G.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
Tissue sample weight	5.0 ± 0.1 g

3. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: One per week per analyst when samples analyzed.

ii. Records are to be maintained.

b. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

i. Investigate following established procedures.

ii. Take corrective action as warranted.

4. Sample Condition upon Receipt – Cool or frozen

J. APPENDIX

Reserved

K. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Staff