

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

SOP No: CLG-FLX4.01		Page 1 of 18
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

**Contents**

A.	INTRODUCTION .....	2
B.	EQUIPMENT .....	2
C.	REAGENTS AND SOLUTIONS .....	4
D.	STANDARDS .....	5
E.	SAMPLE PREPARATION .....	6
F.	ANALYTICAL PROCEDURE.....	7
G.	CALCULATIONS.....	11
H.	SAFETY INFORMATION AND PRECAUTIONS.....	11
I.	QUALITY ASSURANCE PLAN .....	12
J.	WORKSHEET .....	14
K.	APPENDIX .....	16
L.	APPROVALS AND AUTHORITIES.....	18

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

SOP No: CLG-FLX4.01		Page 2 of 18
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

**A. INTRODUCTION**

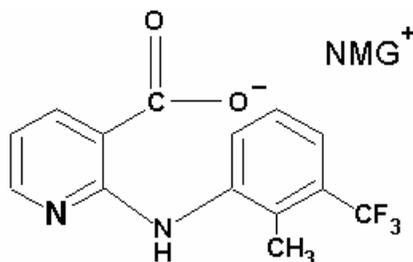
1. Theory

Flunixin, an anti-inflammatory drug present in tissues as flunixin or its acid conjugates, is converted to free flunixin by acid-catalyzed hydrolysis. After neutralizing the acidic mixture, the free flunixin is extracted with ethyl acetate and the extract is purified by passing through a SCX cation exchange cartridge. Flunixin is eluted from the cartridge with ammonium methanol, evaporated to dryness, reconstituted in 50% methanol / water, and quantitated by High Performance Liquid Chromatography/ Electrospray Ionization MS/MS (HPLC/ESI-MS/MS). A matrix-matched external standard curve based on the precursor ion (m/z 297) fragmenting to the product ion m/z 279 is used for quantitation.

2. Applicability

This method is used for determination and confirmation of flunixin in bovine liver at or above 62.5 ppb.

3. Structure



NMG: N-Methylglucamine

**B. EQUIPMENT**

Note: Equivalent equipment may be substituted for the following:

1. Apparatus

- a. Blender - Model No. 51BL31, Waring Inc.
- b. Balance, analytical - Sensitive to 0.1 mg, Model No. A120S, Sartorius.
- c. Balance, top loading - Sensitive to 0.01 g, Model No. E83200D, Shimadzu.
- d. Centrifuge - Capable of 700 x g and holding 50 mL glass centrifuge tubes, Model No. Centra-7R, International Equipment Company.

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

SOP No: CLG-FLX4.01		Page 3 of 18
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

- e. Evaporator - Nitrogen evaporator, N-Evap®, Model No. 111, Organomation.
- f. Glass centrifuge tube - Pyrex® round-bottom screw-cap, 29 mm, 50 mL centrifuge tube, Cat. No. 21023-401, VWR.
- g. Polypropylene centrifuge tubes - 50 mL conical, with closures, Cat. No. 352070, Blue Max.
- h. Glass disposable Pasteur pipettes – 9-inch, Cat. No. 14672-380, VWR.
- i. Glass volumetric flask - Class A, 10, 50, 100, and 1000 mL, Kimax.
- j. Glass graduated cylinder - 500 mL.
- k. Heating Block - Pierce Reach-Therm™ III (Cat. No. 18935 H, Pierce) with three aluminum heating blocks holding at least twelve 29 mm 50 mL centrifuge tubes. The heating block is custom drilled from a blank aluminum block (Cat. No. 18810 H, Pierce).
- l. pH meter - Capable of 0.01 pH unit sensitivity, Model No. 340 pH meter, Corning.
- m. Pipettes - Automatic pipettors capable of accurately delivering 300 µL to 5 mL volume.
- n. Repipettors - Bottle top dispensers of appropriate volume.
- o. Spatula - stainless steel or plastic.
- p. Test tube racks - for 15 and 50 mL tubes.
- q. Solid Phase Extraction Vacuum Manifold - Cat. No. 57030-U, Supelco.
- r. Trap for SPE Vacuum Manifold - Side arm flask with tubing, Cat. No. 57120-U, Supelco.
- s. SCX Cartridges - Benzenesulfonic acid cation exchange (SCX), 500-mg/3 mL, Cat. No. 2323, Applied Separations, Allentown, PA.
- t. Reservoirs - Empty polypropylene SPE tubes (no frits) 60 mL, Cat. No. 57022, Supelco.
- u. Cartridge Adapters - For 3 mL tubes, Cat. No. 57020-U, Supelco.
- v. Disposable glass culture tubes - 15 mL, 16 x 100 mm, Cat. No. 73500 16100, Kimble.
- w. Vortexer - Vortexer-2, and multi-tube vortexer, Cat. No. 58816-115, VWR.
- x. Mobile Phase Filtration Apparatus - Cat. No. 58062-U, Supelco.
- y. Membrane Filter Disks - 0.45 µm pore size, 47 mm, nylon, HPLC mobile phase membrane filter, Cat. No. HNWP 047 00, Fisher.
- z. Whatman Mini-Uniprep syringeless filters - Standard, PTFE, 0.45 µm, Cat. No. 09-923-28, Fisher.

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

SOP No: CLG-FLX4.01		Page 4 of 18
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

2. LC/ESI-MS/MS System

- a. Pumps - Two Varian Prostar model 210 pumps, Master and Slave.
- b. Autosampler with column heating compartment - Varian Prostar model 410 autosampler.
- c. Source - Varian Electrospray ionization.
- d. Detector - Model 1200L quadrupole MS/MS, Varian.
- e. Analytical Column - Eclipse XDB-C18 2.1 x 150 mm, 5  $\mu$ m, Cat. No. 993700-902, Agilent.
- f. Guard Column - Brownlee cartridge column, RPI8, 7  $\mu$ m, 3.2 x 15 mm, Cat. No. 0711-0092 and NewGuard holder (complete), Cat. No. 0715-0001, Chrom Tech.
- g. Data System - MS Workstation version 6.41 with service pack 2, Varian.

**C. REAGENTS AND SOLUTIONS**

Note: Equivalent reagents and solutions may be substituted for the following:

1. Reagents

- a. Ethyl acetate (EtOAc) - HPLC grade, Cat. No. 230-4, Burdick & Jackson.
- b. Methanol (MeOH) - HPLC grade, Cat. No. 100-4, Burdick & Jackson.
- c. Ammonium Hydroxide (NH<sub>4</sub>OH) - 30%, Cat. No. 9721-33, J.T. Baker.
- d. Hydrochloric acid (HCl) - Concentrated, Cat. No. 9535-05, J.T. Baker.
- e. Phosphoric acid (H<sub>3</sub>PO<sub>4</sub>) - 85% in water, Cat. No. 438081-500ML, Sigma.
- f. Formic acid (CH<sub>2</sub>O<sub>2</sub>) - 98 - 100% purity, Cat. No. 27001-500ML-R, Sigma.
- g. Sodium Hydroxide Pellets (NaOH) - Cat, No. 3728-05, J.T. Baker.
- h. Water – Millipore water (deionized distilled).

2. Solutions

- a. 6N Hydrochloric Acid (HCl):  
Mix equal volumes of concentrated hydrochloric acid and water. This solution is stable for one year at room temperature.
- b. 20% Sodium Hydroxide (NaOH):  
Dissolve 200 g of sodium hydroxide into 800 mL of high purity water and mix well. This solution is stable for one year at room temperature.
- c. Methanol / Water (50/50, v/v):  
Mix 500 mL of methanol with 500 mL of water. This solution is stable for six

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

SOP No: CLG-FLX4.01		Page 5 of 18
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

months at room temperature.

d. 0.1% Phosphoric Acid / Methanol:

Pipet 1 mL of phosphoric acid into a 1 L volumetric flask. Fill to volume with methanol. This solution is stable for one year at room temperature.

e. Ammonium Hydroxide / Methanol (10/90 v/v):

Mix 20 mL of 28-30% ammonium hydroxide solution with 180 mL of methanol. This solution is stable for 6 months at room temperature.

f. 1% Formic Acid / Methanol:

Pipet 1 mL of formic acid into a 100 mL volumetric flask. Fill to volume with methanol. This solution is stable for 6 months at room temperature.

g. HPLC Mobile Phase:

i. Aqueous Mobile Phase (0.4% formic acid in water):

Pipet 4 mL of formic acid into a 1 L volumetric flask. Fill to volume with Millipore water. Mix and filter through a 0.45 µm nylon filter. This solution is stable for 1 month at room temperature.

ii. Organic Mobile Phase (0.18% formic acid in 4:5 acetonitrile:methanol):

Pipet 1.6 mL of formic acid into a 1 L bottle. Add 400 mL acetonitrile and 500 mL methanol. Mix and filter through a 0.45 µm nylon filter. This solution is stable for 1 month at room temperature.

## D. STANDARDS

Note: The analyst may prepare different standard volumes and/or concentrations to cover the range of interest.

### 1. Reference Standard

Common Name: Flunixin-N-methyl glucamine salt (NMG), or flunixin meglumine

Chemical Name: 2-[[2-Methyl-3-(trifluoromethyl)-phenyl]-amino]-3-pyridinecarboxylic acid (as NMG salt), C<sub>21</sub>H<sub>28</sub>F<sub>3</sub>N<sub>3</sub>O<sub>7</sub>.

Molecular Weight: 491 (Free Acid MW is 296).

Source: Cat. No. SCH 14714, Schering-Plough, Union, NJ and Cat. No. 27460-7, U.S. Pharmacopoeia, Rockville, MD.

Storage: At room temperature.

### 2. Preparation of standard solutions

Note: If the purity is less than 100%, make corrections based on the actual purity provided.

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

SOP No: CLG-FLX4.01		Page 6 of 18
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

- a. Stock Solution (500 µg/mL as free acid):  
Weigh 8.3 mg flunixin-NMG analytical standard equivalent to 5.0 mg of flunixin free acid into a 10 mL volumetric flask, dissolve the material, and dilute to the mark with methanol. This stock standard is stable for 6 months at less than -10 °C.
- b. Intermediate Stock Solution (5 µg/mL as free acid):  
Pipet 500 µL of the stock solution into a 50 mL volumetric flask and dilute to the mark with methanol. This stock standard is stable for 6 months if stored at less than -10 °C.
- c. Fortification Solution (250 ng/mL as free acid):  
Pipet 5 mL of the intermediate stock solution (5 µg/mL) into a 100 mL volumetric flask. Dilute to the mark with 50% methanol / water. Fortification solution stored in the freezer at less than -10 °C is stable for six months.
- d. LC/MS Matrix-Matched Standard Curve Solutions:  
Weigh 2 g of blank tissue and carry through hydrolysis and extraction steps F.1.a - i. Clean up four 10 mL aliquots of the resulting extract using steps F.1.k - l. Add 0, 125, 250, and 500 µL of Fortification standard solution to tubes and reconstitute with 50:50 methanol / water, as shown in the following Table below:

LC/MS STD Calibration Level	Vol. Fortification Solution, µL	Vol. 50:50 MeOH/water (µL)	Flunixin free acid (ng/mL)	ppb equivalent
0	0	2000	0	0
1	125	1875	15.6	62.5
2	250	1750	31.3	125
3	500	1500	62.5	250

Vortex and filter according to steps F.1.n. – o. below.

3. Storage and Stability

Stored in the refrigerator at 2 - 8 °C these solutions are stable for five months.

**E. SAMPLE PREPARATION**

Cut entire liver into chunks and thoroughly homogenize using a food grinder. Store liver at less than -10 °C when not processing.

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

SOP No: CLG-FLX4.01		Page 7 of 18
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

**F. ANALYTICAL PROCEDURE**

**1. Sample Extraction and Cleanup**

- a. Weigh  $2.00 \pm 0.10$  g of tissue homogenate into a 50 mL round-bottom screw top centrifuge tube. Record the weights to three significant figures.

Note: Fortification of recovery should be performed during this step along with a blank. For a 125 ppb recovery, fortify 2 g of control liver with 1000  $\mu$ L of fortification solution (D.2.c.). Label the tube and cap to prevent possible cross contamination.

- b. In a well-ventilated hood, add 8 mL of 6N HCl to each tube. Using a spatula, scrape the tissue down into the liquid.
- c. Place the sample tubes in a heating block preheated to 110 -120 °C. The temperature of the heating block will gradually drop to ~95 °C when the tubes are in the heating block. The temperature will rise to 110 -120 °C within 15 min. After at least 2 hours of heating at ~95 -120 °C, allow cooling to ambient temperature. Tubes can be immersed into a cold water bath to accelerate the cooling process.

STOPPING POINT – Samples may be stored at 2 -8 °C up to 3 days.

- d. Adjust the pH of the HCl hydrolysate to pH 9.5 (9.30 - 9.70) by slowly adding approximately 6 mL of 20% NaOH and gently mixing. Check the pH of the solution with a pH meter and add additional 20% NaOH or 6N HCl if necessary. Rinse the pH meter probe with water between samples.
- e. Add 10 mL of ethyl acetate to each tube, cap and vortex at high speed for 1 min. (Alternatively, shake the test tube rack along the long axis of the tubes for 5 minutes).
- f. Centrifuge the tubes for 5 min at approximately 700 g.
- g. Using a long-stem Pasteur pipette, transfer the upper ethyl acetate extract to another properly labeled, clean 50 mL polypropylene centrifuge tube. Care should be taken not to transfer any of the lower dark layers.
- h. Repeat the 10 mL ethyl acetate solvent partition three more times and combine the ethyl acetate fractions (step g.) and adjust the volume to 40 mL with ethyl acetate using the graduations on the tube. Discard aqueous layer.
- i. Cap the tube and mix thoroughly.  
STOPPING POINT: Cap the tubes. Extracts are stable up to three days at 2 -8 °C.
- j. Precondition 15 mL disposable glass culture tubes by vortexing the tubes containing 2 mL of a 1% formic acid in methanol for about 30 sec, pouring out the residual solution, and allowing the tubes to dry.

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

SOP No: CLG-FLX4.01		Page 8 of 18
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

- k. SCX Cartridge Cleanup
    - i. Attach 60 mL reservoirs to SCX column cartridges on a vacuum manifold.
    - ii. Condition the cartridges as follows:
      - (a) Add 6 mL water and allow to drain to near the bed surface. Do not allow column to run dry.
      - (b) Add 6 mL 0.1% phosphoric acid in methanol and allow to drain until 1 mL remains above the bed.
    - iii. Transfer 10 mL of ethyl acetate extract from step (F.1.i) into the 60 mL reservoir and mix with an equal volume of 0.1% phosphoric acid in methanol and let the mixture flow through the SCX cartridge. Do not allow the cartridge to run dry.
    - iv. Rinse the cartridge with 5 mL of ethyl acetate followed by 5 mL of methanol. Discard the eluate.
    - v. Elute the residue with 12 mL of NH<sub>4</sub>OH (28-38%) / MeOH (10/90, v/v) into a preconditioned 15-mL disposable glass culture tube (j) at about 1 drop per minute. Ensure complete elution by observing only air passing through the column.
  - l. Evaporate the NH<sub>4</sub>OH / MeOH eluate to dryness under a stream of nitrogen or air using an N-Evap<sup>®</sup> in a < 70 °C water bath.
  - m. Reconstitute the residue in 2 mL of 50% methanol / water to yield the final extract.
  - n. Vortex for approximately 10 seconds.
  - o. Filter a portion of the extract to an autosampler vial for LC/MS/MS analysis using 0.45 µm PTFE filters.

STOPPING POINT – The procedure may be stopped at this point. Samples may be stored at 2 - 8 °C for up to 5 months.
2. LC/MS Analysis
- a. Instrument Settings

Note: The following instrument parameters may be optimized:

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

SOP No: CLG-FLX4.01		Page 9 of 18
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

i. HPLC Mobile Phase Gradient Table:

Run time (min)	Flow rate (mL/min)	(A) Aqueous (%)	(B) Organic (%)	Switching valve
0:00	0.40	55	45	Waste
9:00	0.40	55	45	MS
13:00	0.40	55	45	Waste
13:30	0.40	5	95	
13:50	0.55	5	95	
17:50	0.55	5	95	
18:00	0.55	55	45	
23:00	0.55	55	45	
23:10	0.40	55	45	

vi. Mass Spectrometric Parameters:

Nebulizing gas pressure	50 psi
Needle voltage	5000 V
Shield voltage	600 V
Housing temperature	50 °C
Drying gas temperature	400 °C
Drying gas pressure	30 psi
Capillary voltage	44 V
Manifold temperature	42 °C
CID gas pressure	2 mTorr
Detector voltage	1800 V
Injection volume	50 µL
Column temperature	40 °C
Ion mode	Positive
Precursor ion m/z	297
Product ions m/z	279, 264, 259

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

SOP No: CLG-FLX4.01		Page 10 of 18
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

Quantitate using the signal area from the precursor ion fragmenting to the most abundant product ion (m/z 279) using multiple reaction monitoring.

- b. Analyze Sample Set:
  - i. Create a calibration curve using the matrix-matched external standards at 0, 62.5, 125, and 250 ppb, respectively. The correlation coefficient must be  $\geq 0.995$ .
  - ii. The signal to noise ratio of the standard equivalent to 125 ppb must be  $> 20$ .
  - iii. Analyze a solvent blank, a tissue blank, a recovery, and a check sample (one per week per analyst), and samples. No carry-over should be observed in the solvent blank.
- c. Confirmation Criteria
  - i. The tissue blank has no confirmable flunixin.
  - ii. The retention time of the unknown must be  $\pm 3\%$  of the matrix-matched external standards.
  - iii. All three ion products (m/z = 279, 264, 259) must be present.
  - iv. The ion ratios 259/279 and 264/279 must match those of the matrix-matched external standard within a relative difference of  $\pm 20\%$ .
3. Sample Chromatogram and Mass Spectrum  
(See Appendix 2).
4. Proposed fragmentation pattern of flunixin  
(See Appendix 3).

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

SOP No: CLG-FLX4.01		Page 11 of 18
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

**G. CALCULATIONS**

1. Using linear regression analysis, calculate the slope, intercept, and correlation coefficient of a standard curve constructed by plotting peak areas versus concentration (ng/mL) for all the injected standards. The correlation coefficient must be  $\geq 0.995$ .

2. The concentration of flunixin can be calculated using the following equation:

$$\text{ppb flunixin} = \frac{(A - B) \times D}{C \times E} \quad \text{where}$$

A = flunixin peak area.

B = intercept from the calibration curve.

C = slope of the calibration curve.

D = volume (mL) = (ethyl acetate extract volume / aliquot volume) x final volume = (40 mL / 10 mL) x 2 mL = 8 mL.

E = weight of the tissue sample.

3. Calculate results when the following conditions are met:

- a. The correlation coefficient for the standard curve is  $\geq 0.995$ .
- b. The recovery of the positive control falls within the limits specified in section I.1.

**H. SAFETY INFORMATION AND PRECAUTIONS**

1. Required Protective Equipment — Safety glasses, disposable gloves, lab coat.
2. Hazards

Procedure Step	Hazard	Recommended Safe Procedures
F.1.a.	Cracked and damaged tubes	Cracked or damaged tubes should be discarded.
Acid hydrolysis F.1.b-c.	Closed systems under high temperatures and pressures with corrosive liquids and gases.	Use 6N HCl only in a fume hood. Recommend safety shield or hood sash for the hydrolysis.
Acid neutralization F.1.d.	Strong acids and bases are corrosive. The neutralization is an exothermic reaction which may spatter.	Care must be used to avoid spattering of samples. Conduct this procedure under a fume hood. An ice/water bath may be

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

SOP No: CLG-FLX4.01		Page 12 of 18
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

used during neutralization.

Reagents / Solutions	Hazard	Recommended Safe Procedures
Ammonium hydroxide, hydrochloric acid, phosphoric acid, formic acid, sodium hydroxide	Corrosive, burns	Wear protective equipment. Avoid contact with skin. Work in a fume hood.
Methanol and ethyl acetate	Flammable and poisonous	Keep in well-closed containers in a cool place and away from fire. Work in a fume hood.

3. Disposal Procedures

Reagents / Solutions	Hazard	Recommended Safe Procedures
Methanol and ethyl acetate mixed waste, LC mobile phase	Flammable and poisonous	Collect in a tightly sealed container and store away from non-compatibles. Dispose in accordance with local, state, and federal regulations.

**I. QUALITY ASSURANCE PLAN**

1. Performance Standard

Analyte	Analytical Range	Recovery
Flunixin	62.5 – 250 ppb	60 – 110%

2. Critical Control Points and Specifications

Record	Acceptable Control
a. Sample weight	2.0 ± 0.1 g.
b. Acid hydrolysis time	≥ 2 hours.
c. Acid hydrolysis temperature	95 - 120 °C.
d. pH adjusted to	pH 9.5 ± 0.2.

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

SOP No: CLG-FLX4.01		Page 13 of 18
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

3. Readiness To Perform (FISIS Training Plan)

a. Familiarization

- i. Phase I: Standards - Analyze in duplicate matrix-matched external standard curve solutions at 0, 62.5, 125, and 250 ppb (D.2.d.) on each of 3 days to verify instrument response (F.2.b.i.).
- ii. Phase II: Fortified samples – Analyze 2 replicates at 0, 62.5, 125, and 250 ppb over a period of 3 different days.

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

- iii. Phase III: Check samples for analyst accreditation given by the supervisor or designee.
  - (a) Eight unknown samples, at least one of which is a blank.
  - (b) Report analytical findings to three significant figures.
  - (c) Letter from Quality Assurance Manager (QAM) is required to commence official analysis.

b. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

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

4. Intralaboratory Check Samples

a. System, minimum contents.

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

b. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

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

5. Sample Acceptability and Stability

- a. Matrix: Bovine liver.
- b. Sample receipt size: approximately 500 g.
- c. Condition upon receipt: not spoiled or rancid.

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

SOP No: CLG-FLX4.01		Page 14 of 18
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

- d. Sample storage:
  - i. Time: 2 months.
  - ii. Condition: frozen < -10 °C.
  
- 6. Sample Set
  - a. Matrix-matched external standards for calibration curve (blank plus 3 levels).
  - b. One tissue blank.
  - c. One 125 ppb recovery.
  - d. One intra-laboratory check sample per week per analyst.
  - e. Samples.
  
- 7. Sensitivity  
Minimum proficiency level (MPL): 62.5 ppb.

**J. WORKSHEET**

The following Worksheet is an example:

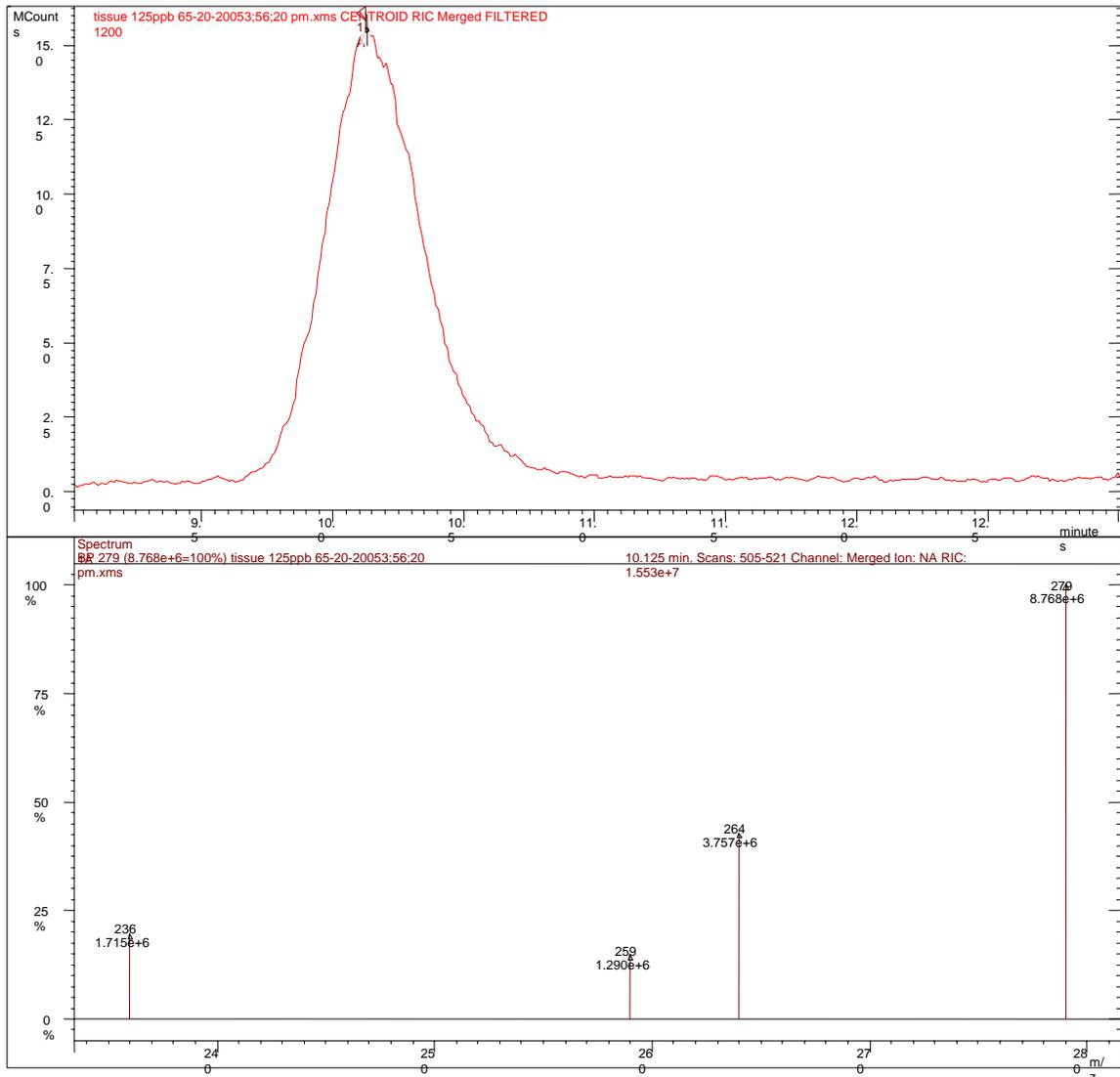


**United States Department of Agriculture  
Food Safety and Inspection Service, Office of Public Health Science**

SOP No: CLG-FLX4.01		Page 16 of 18
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

**K. APPENDIX**

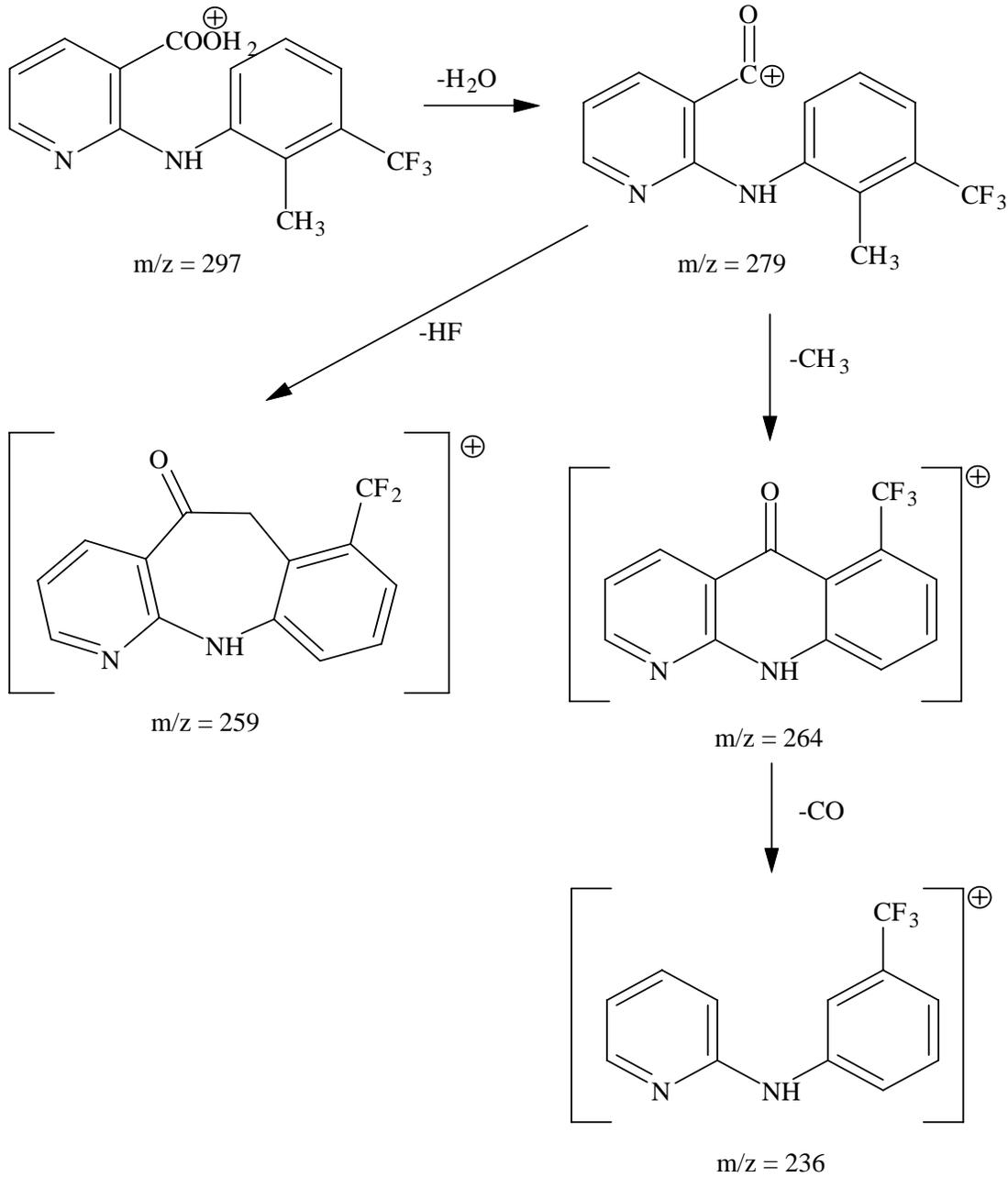
1. Reference: Boner, Pamela L. et al, J. Agric. Food Chem. 2003, 51, 7555-7559.
2. Reconstituted ion chromatogram and spectrum for bovine liver fortified with 125 ppb flunixin.



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

SOP No: CLG-FLX4.01	Page 17 of 18	
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

3. Proposed fragmentation pattern of flunixin.



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

SOP No: CLG-FLX4.01		Page 18 of 18
Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: .01	Replaces: .00	Effective: 02/21/2006

**L. APPROVALS AND AUTHORITIES**

1. Approved by:

In Suk Kim

David Martin

Jess Rajan

Phyllis Sparling

\*Charles Pixley

*Approvals on file.*

2. \*Issuing Authority: Laboratory Quality Assurance Division (LQAD).