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Food Safety and Inspection Service, Office of Public Health Science**

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Title: Confirmation of Phenylbutazone by LC/ESI-MS/MS		
Revision: 03	Replaces: CLG-PBZ2.02	Effective: 12/10/2007

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**A. INTRODUCTION**

1. Theory

Phenylbutazone (PBZ) is extracted from kidney tissues with aqueous ammonium hydroxide. The extract is partitioned with mixed ethers to remove lipids, then acidified, and PBZ is extracted with a tetrahydrofuran (THF)-hexane solution. This extract is further purified by silica gel solid phase extraction (SPE). The eluate is evaporated, then redissolved in mobile phase and analyzed by HPLC/Electrospray ionization MS/MS (HPLC-ESI-MS/MS) in positive ion multiple reaction monitoring (MRM) mode. Confirmation is based on comparison of HPLC retention times and MS/MS daughter ion ratios against those from a reference standard.

2. Applicability

This method is applicable for confirmation of phenylbutazone in bovine kidney at  $\geq 100$  ppb.

**B. EQUIPMENT**

Note: Equivalent equipment may be substituted for the following.

1. Apparatus

- a. Centrifuge - IEC model B 22 M Super-speed Refrigerated Centrifuge with Rotor No. 876 capable of attaining 5000 rpm (3140 rcf), (Refrigeration is not required), Cat. No. 20671-007, VWR Scientific.
- b. Eppendorf pipettors - Variable volume pipettes: 2 - 20  $\mu$ L (Cat. No. 05-402-46), 10 - 100  $\mu$ L (Cat. No. 05-402-48), 50 - 200  $\mu$ L (Cat. No. 05-402-49), 100 - 1000  $\mu$ L (Cat. No. 05-402-50) and 500 - 2500  $\mu$ L (Cat. No. 05-402-51) Fisher Scientific.
- c. SPE columns - Silica solid phase extraction columns (6 mL, 500 mg), Part No. 43400, Waters Corp.
- d. Nitrogen Evaporator - Organomation Associates.
- e. Vortex mixer - Fisher Scientific.
- f. Balance - PM 300, Mettler.
- g. Polyvinylidene fluoride (PVDF) membrane filter - 0.2  $\mu$ m, Product No. 4455, Pall Life Sciences. (Optional; for filtering mobile phase.)
- h. Syringeless Filter Device - Mini-UniPrep with nylon filter media, 0.45  $\mu$ m pore size, Cat. No. UN203NPUNYL, Waters. (Optional: PVDF filter media may be substituted for nylon.)
- i. Glassware - Volumetric Glassware includes 10 mL, 25 mL, 50 mL, 100 mL, and 250 mL graduated cylinders.
- j. Test tubes - 16 mm x 125 mm (20 mL) disposable Borosilicate glass culture tubes, Kimble.
- k. Test tubes - 50 mL polypropylene tube with polypropylene screw closure, Cat. No. 3139-0050, Nalge Company.

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- i. SPE vacuum manifolds - Supelco.
    - m. Shaker - Eberbach Corporation.
    - n. Food processor - Robot Coupe model RSI6Y-1, Robot Coupe USA Inc.
  2. Instrumentation
    - a. Mass spectrometer - Waters Micromass Quattro Micro API triple quadrupole.
    - b. HPLC - Waters Alliance 2695.
    - c. Analytical column - YMC ODS-AQ, 120 Å, 2 x 100 mm, 3 µm, Part No. AQ 12S031002 WT, Waters Corp.

**C. REAGENTS AND SOLUTIONS**

Note: Equivalent reagents/solutions may be substituted for the following.

1. Reagents
  - a. Ethyl ether - Reagent grade, Cat. No. 107-1, Burdick & Jackson.
  - b. Petroleum ether - Reagent grade, Cat. No. 317-4, Burdick & Jackson.
  - c. Tetrahydrofuran (THF) - Reagent grade, Cat. No. 340-2, Burdick & Jackson.
  - d. Hexane - Reagent grade, Cat. No. H303-4, Fisher Scientific.
  - e. Acetonitrile (ACN) - HPLC grade, Cat. No. AH015-4, Burdick & Jackson.
  - f. Water - Deionized water, HPLC Grade, Millipore Rx system.
  - g. Methanol (MeOH) - HPLC grade, Cat. No. AH230-4, Burdick & Jackson.
  - h. Ammonium Hydroxide (NH<sub>4</sub>OH) - 30% Cat. No. 9721-01, J. T. Baker.
  - i. Hydrochloric acid (HCl) - Reagent grade, Cat. No. A144-212, Fisher Scientific.
  - j. Formic acid - Mass spectrometric grade, Cat. No. 94318, Fluka through Sigma-Aldrich.
2. Solutions
  - a. 7.5% Ammonium Hydroxide Solution:  
Dilute 25 mL of 30% ammonium hydroxide to 100 mL with deionized water using a graduated cylinder.
  - b. LC Aqueous Mobile Phase: 0.1% formic acid in water:  
Add 1 mL of formic acid to a 1 L class A volumetric flask. Dilute to volume with fresh Millipore water. Mix well. Either sonicate to degas or filter before use.
  - c. THF/Hexane (1:4):  
Add 60 mL of THF to 240 mL of hexane. Mix well.
  - d. Solvent 1 - acetonitrile:0.2N acetic acid (50:50):

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Dilute 3 g or 2.9 mL of conc. acetic acid (17.4N) to 250 mL with deionized water. Add this solution to 250 mL acetonitrile. Mix well.

e. 6N HCl:

Dilute conc. (12N) HCl 1 to 1 with deionized water.

#### **D. STANDARDS**

Note: Equivalent standard/solutions may be substituted for the following.

1. Source

Phenylbutazone (C<sub>13</sub>H<sub>20</sub>N<sub>2</sub>O<sub>2</sub>, MW 308 and CAS 50-33-9) standard, Cat. No. 53567, ICN Biomedical Inc..

2. Preparation of Standards

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

a. Stock PBZ Standard Solution (500 µg/mL):

Accurately weigh 50.0 ± 0.1 mg PBZ standard into a 100 mL volumetric flask. Dissolve and bring to volume with methanol. Stable for two months at 2 - 8 °C.

b. PBZ Working Standard (5 µg/mL):

Dilute 50 µL of 500 µg/mL stock standard (D.2.a.) solution to 5 mL with methanol in a volumetric flask.

Note: This solution is stable for two months if stored at 2 - 8 °C.

c. LC/MS/MS External Standards (100 ng/mL):

Dilute 20 µL of 5 µg/mL standard solution (D.2.b) with 980 µL of solvent 1 (C.2.e.) in a LC sample vial.

#### **E. SAMPLE PREPARATION**

After removing excessive fat from kidney sample, cut kidney into smaller pieces and homogenize to fine paste with a mechanical food processor. Transfer homogenized sample into plastic bags and store in the freezer at -20 °C. Let the sample partially thaw prior to analysis.

#### **F. ANALYTICAL PROCEDURE**

1. Sample extraction and cleanup procedure

a. Weigh 5.0 ± 0.1 g of thawed homogenized kidney sample into a 50 mL polypropylene centrifuge tube.

Prepare positive and negative controls as follows:

i. Weigh 5.0 ± 0.1 g blank tissues into two 50 mL polypropylene centrifuge tubes.

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- ii. Prepare the 100 ppb positive control by fortifying one of the tubes with 100  $\mu$ L of 5  $\mu$ g/mL PBZ working standard (D.2.b.). Vortex vigorously for 10 sec.
- b. Add 5 mL deionized water and 0.5 mL ammonium hydroxide solution (C.2.a.) to tube.
- c. Vortex vigorously for 20 sec.
- d. Add approximately 10 mL ethyl ether to tube, cap, and vortex for 10 sec.
- e. Add 10 mL petroleum ether to tube, cap, and shake vigorously for 30 sec.
- f. Centrifuge tube at 3140 rcf at room temperature for 5 min or until layers separate.
- g. Remove and discard top ether layer using a Pasteur pipette.
- h. Vortex tissue and aqueous layer to homogenize.
- i. Add 1 mL 6N HCl (C.2.f.) to each tube, cap and vortex for 30 sec to ensure that the acid was incorporated throughout the tissue mixture.
- j. Add 20 mL THF/hexane (C.2.d.) to tube, cap and mix vigorously on a shaker for 10 min.  
  
STOPPING POINT - If sample extraction is not completed on the same day, store below 8 °C.
- k. Centrifuge at 3140 rcf at room temperature for 10 min or until layers separate.
- l. Prepare silica SPE cartridge by passing 3 mL of THF/hexane (C.2.d.).  
Note: Do not let the SPE column go dry after preparation step.
- m. Pass the first 10 mL of the upper organic layer from sample tube through the prepared SPE cartridge and collect the eluate in a 16 X 125 mm test tube.  
Optional: Stop the SPE and switch to a new 16 X 125 mm test tube.  
Transfer the rest of the upper organic layer from the centrifuge tube to the SPE and collect eluate.  
  
(Discard remaining contents of the centrifuge tube appropriately.)  
  
Note: Do not let the SPE column go dry at any step during elution or rinsing.
- n. Rinse the cartridge with 3 mL THF/hexane (C.2.c.) and collect this eluate in the 16 X 125 mm test tube along with the eluate from step F.1.m.
- o. Evaporate the eluate using a gentle stream of nitrogen in an approximately 50 °C water bath. If using the entire organic layer (optional step in F.1.m.), combine the two eluates once they have evaporated approximately halfway and continue evaporation to dryness.
- p. Dissolve residue in 500  $\mu$ L solvent 1 (C.2.e.) by vortexing for 1 min. If using the entire organic layer (optional step in F.1.m.), use 1 mL of solvent 1.
- q. Filter through a 0.45  $\mu$ m Whatman mini-uniprep filter device.

Note: For a 100 ppb recovery, the concentration of phenylbutazone in the above filtrate will be 500 ng/mL.

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r. The sample is ready for HPLC/ESI-MS/MS analysis.

Note 1: If necessary, the sample extract may be diluted to better approximate the concentration of external standard or fortified control.

Note 2: Stopping point: If the sample extract is not analyzed on the same day, store in a -20 °C freezer.

2. Instrumental Parameters

Note: The instrument parameters listed here are typical values for the instrument specified. The analyst should optimize parameters for the specific instrument being used.

a. HPLC conditions:

Column temperature	25 °C
Aqueous Mobile Phase	0.1% formic acid in water
Organic Mobile Phase	Acetonitrile
Flow rate	0.25 mL/min
Injection volume	10 µL
Run Time	10 min

b. HPLC Mobile Phase Gradient Table:

Time (min)	% Aqueous	% Organic
0	50	50
1	50	50
5	5	95
5.1	50	50
10	50	50

c. Interface and MS conditions:

Mode of operation	Positive Ion Mode
Source temperature	125 °C
Desolvation temperature	450 °C
Cone gas flow	5 L/hr
Desolvation gas flow	750 L/hr
Capillary voltage	1 kV
Cone voltage	30 V
Multiplier voltage	650 V

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- d. MRM Parameters:
- |                    |                   |
|--------------------|-------------------|
| Precursor ion:     | 309 m/z           |
| Product ion (m/z): | Collision energy: |
| 120                | 20 eV             |
| 160                | 21 eV             |
| 188                | 16 eV             |

**G. CONFIRMATION**

- The retention time of the peak must match that of the comparison standard (either the external standard or the fortified control) within 5%.
- The peak of interest should exceed a signal to noise (S/N) ratio of 3:1.
- LC/MS/MS Criteria

Ion ratios are measured using the most abundant product ion in the comparison standard as the base peak. Specific criteria are as follows:

- The product ion ratio using two of the product ions must be within 10% absolute difference of the comparison standard.
- Alternatively, two product ion ratios using three of the product ions must be within 20% absolute difference of the comparison standard.

The criteria for comparison of ion ratios must be consistently applied to all of the samples in a set.

Ion ratios used:

	Ratio #1	Ratio #2
PBZ	160 / 120	188 / 120

Note: The ion ratios in the above chart reflect those used for the instrumentation listed in Section B.2. Equivalent instrumentation may measure different ratios using any of the product ions listed in Section F.2.d.

- The following injection sequence may be used for sample analysis:
  - External standard
  - Solvent blank
  - Negative tissue control (blank tissue)
  - Positive tissue control (recovery)
  - Samples

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f. External standard or fortified control

Note: Carryover may be observed after a high concentration of phenylbutazone is analyzed. It is recommended that a solvent blank is injected after a sample or standard with a high phenylbutazone concentration.

5. The negative control must be negative for the analyte using criteria in G.1. - G.3.
6. The positive control must be positive for the analyte using criteria in G.1. - G.3.

**H. SAFETY INFORMATION AND PRECAUTIONS**

1. Required Protective Equipment - Safety glasses, disposable gloves, lab coats.
2. Hazards

Reagents / Solutions	Hazard	Recommended Safe Procedure
Methanol, diethyl ether, petroleum ether, tetrahydrofuran.	Flammable and poisonous  Diethyl ether and tetrahydrofuran can form explosive peroxides after extended exposure to air.	Wear gloves, work in fume hood.  Do not allow accumulations of diethyl ether or tetrahydrofuran dry out.
HPLC mobile phase containing formic acid, acetonitrile, and water.	Irritation to skin, eyes, nose, mouth, throat and mucous membrane and may cause burns to skin.	Wear gloves, work in hood. Use protective eyewear.

3. Disposal Procedures

Reagents / solutions	Hazard	Recommended Safe Procedure
Methanol, diethyl ether, petroleum ether, tetrahydrofuran.	Flammable and poisonous  Diethyl ether and tetrahydrofuran can form explosive peroxides after extended exposure to air.	Collect in a tightly sealed container and store in the flammable liquid storage area for disposal in accordance with local, state, and Federal regulations.
HPLC mobile phase containing formic acid, acetonitrile, and water	Irritation to skin, eyes, nose, mouth, throat and mucous membrane and may cause burns to skin.	Neutralize and transfer waste in a tightly sealed container and store away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local,

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state, and Federal regulations.

**I. QUALITY ASSURANCE PLAN**

1. Performance Standard

- a. No false positives from blank tissues.
- b. No false negatives from recoveries fortified at 100 ppb.

2. Critical Control Points and Specifications

*Record*

Reconstituted extract

*Acceptable Control*

Filter prior to the injection into HPLC-MS for analysis.

3. Readiness to Perform

a. Familiarization

- i. Phase I, Standard(s): Prepare and analyze a 100 ppb phenylbutazone standard solution and a solvent blank..Evaluate using criteria stated in section G. Repeat on three different days to verify that instrument response is adequate for confirmation of the analyte.
- ii. Phase II, Analyst fortified sample extracts: Extract and analyze two blank tissues and two 100 ppb PBZ fortified tissue recoveries on three different days.  
  
Note: Phases I and II can be performed concurrently.
- iii. Phase III, Check samples for analyst accreditation:
  - (a) A minimum of 8 blind check samples. At least one check sample should be blank. Samples should be fortified at the 100 ppb level.
  - (b) Report analytical findings to Supervisor/Quality Assurance Manager (QAM).
  - (c) Notification from the QAM is required to commence official sample analysis.

4. Intralaboratory check samples

a. System, minimum contents.

- i. Frequency: 1 per week for each analyst when samples are analyzed.
- ii. Records are maintained.

b. Acceptability criteria: Refer to section I.1 above.

If unacceptable results are obtained, then:

- i. Stop all sample analysis by the analyst.

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- ii. Take corrective action.
5. Sample set must include:
- a. External standard.
  - b. Positive control (fortified tissue).
  - c. Negative control (tissue blank).
  - d. Sample extract(s).
6. Sensitivity  
Minimum Proficiency Level (MPL): 100 ppb.

**J. WORKSHEET**

The following worksheet is an example.

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**Phenylbutazone LC/MS/MS Worksheet**

Analyst	
Start Date	
End Date	
Peer Review/Date	
Supervisor Review/Date	

Sample Freezer	
Balance	
Vortex / Shaker	
Centrifuge	
Timer	
SPE Lot #	
Evaporator (approx 50 °C)	
Were samples filtered?	yes / no

Reagents	(Volume)	ID	Pipet/Disp
DI Water	(5 mL)		
NH <sub>4</sub> OH	(500 uL)		
Ethyl Ether	(10 mL)		
Pet. Ether	(10 mL)		
6 N HCl	(1 mL)		
THF/Hexane (20 / 3 / 10 mL)			
Solvent 1 (C.2.e.) (500 uL)			
Aqueous Mobile Phase			N/A
Organic Mobile Phase			N/A

Standards	ID	Pipet
5 µg/mL (working std)		
100 ng/mL (external std)		N/A

Sample	ID	Weight (5.0 ± 0.1 g)
Blank		
Rec		
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
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LC/MS Notebook	
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Comments

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<b>Analyst</b>	
<b>PeerReview</b>	
<b>Supervisory Review</b>	

**Primary Instrument -  
Raw Data for Standards and Samples With Observed Ion Peaks**  
(Comparison standard may be external standard or fortified tissue)

Sample	Retention Time	120 Ion Area	160 Ion Area	188 Ion Area
Comparison Standard				

**Ion Ratios (as Percent of Base Peak)**

Sample	Ratio 1 (160/120)	Ratio 2 (188/120)
Comparison Standard		

**Absolute Percent Difference from Comparison Standard**

Sample	Retention Time	Ratio 1 (120/160)	Ratio 2 (188/160)

(Confirmation requires RT within 5% and one ion ratio within 10% or two ion ratios both within 20% of standard)

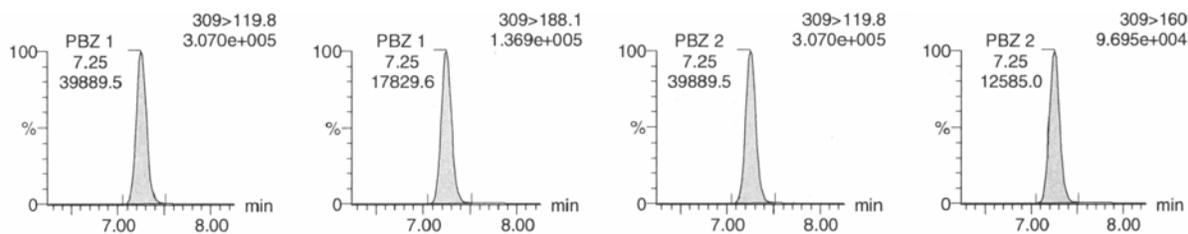
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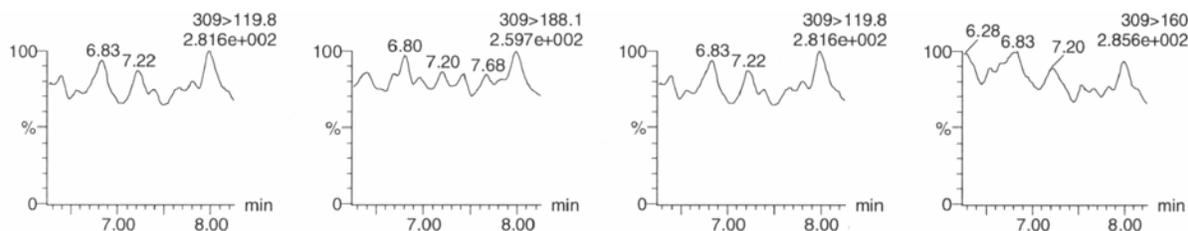
**K. APPENDIX**

1. Chromatograms

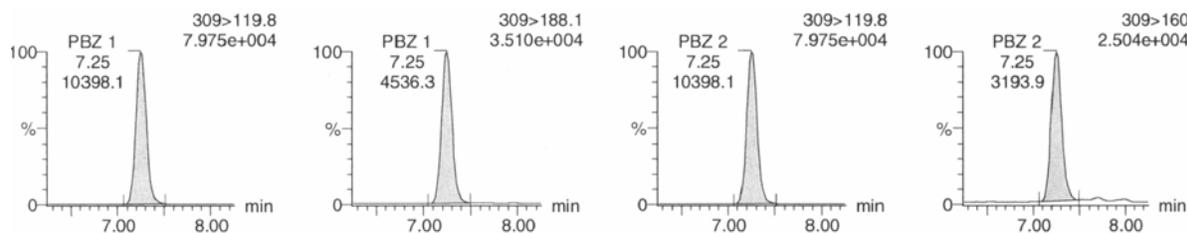
a. Chromatograms of 100 ppb phenylbutazone external standard.



b. Chromatograms of negative control (unfortified bovine kidney).



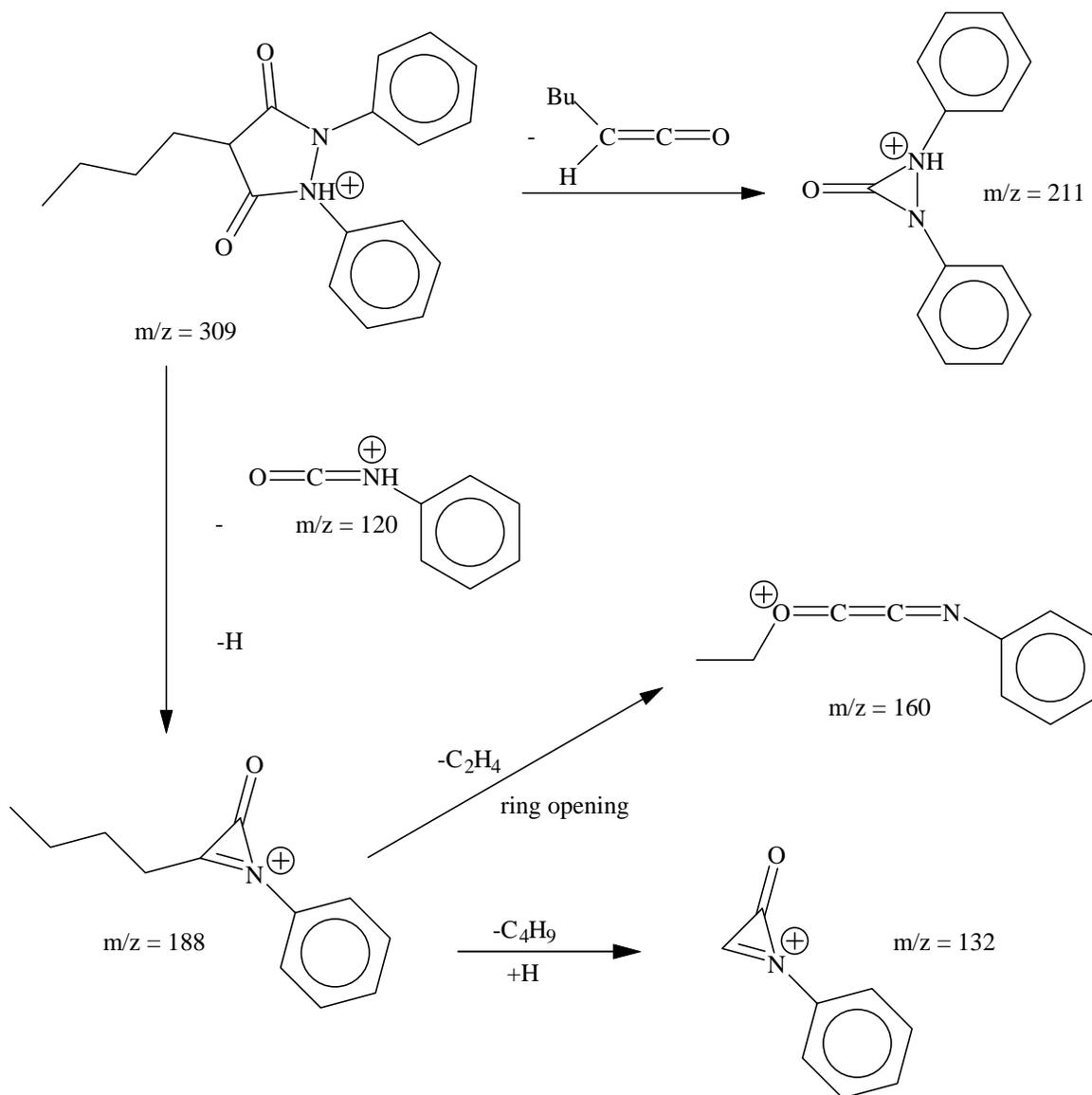
c. Chromatograms of positive control (100 ppb fortified bovine kidney).



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2. Proposed fragmentation pattern of phenylbutazone



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3. Reference

Susan B. Clark, Sherri B. Turnipseed, Gene J. Nandrea, Mark R. Madson, Jeffrey A. Hurlbut and John N. Sofos. Confirmation of Phenylbutazone Residues in Bovine Kidney by Liquid Chromatography/Mass Spectrometry, J. AOAC Int. (2002) 85 (5), 1009 - 1014.

**L. APPROVALS AND AUTHORITIES**

Approvals are on file.

Issuing Authority: Laboratory Quality Assurance Division (LQAD).