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Title: Screening and Confirmation of Thyreostats by HPLC/MS/MS		
Revision: 01	Replaces: CLG-TST2.00	Effective: 01/22/2009

Contents

A. INTRODUCTION

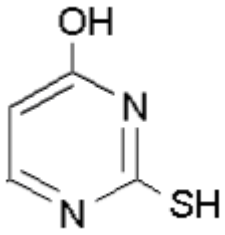
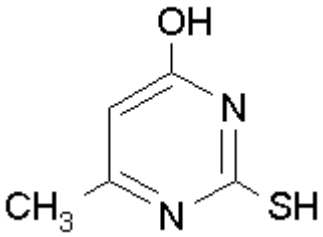
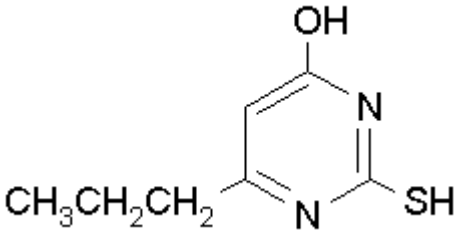
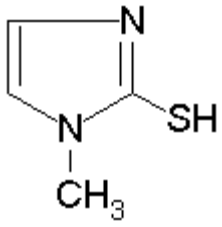
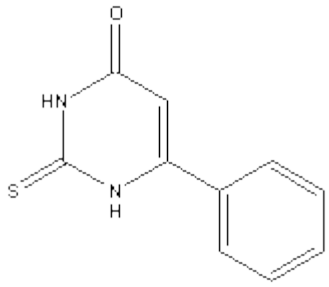
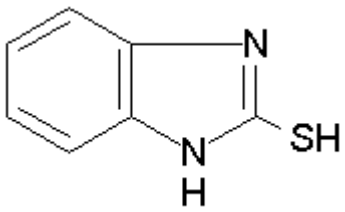
1. Theory

Thyreostat residues are extracted with acetonitrile from muscle homogenate. The extract is partially cleaned by passing through the silica gel column, and then analyzed by HPLC-MS/MS for confirmation. Confirmation is based on comparison of sample MS/MS spectral data with that of a fortified tissue standard or external standard.

2. Applicability

This procedure is applicable for the screening and confirmation of thyreostats (2-thiouracil, 6-methyl-2-thiouracil, 6-propyl-2-thiouracil, 6-phenyl-2-thiouracil, 2-mercapto-1-methylimidazole, and 2-mercaptobenzimidazole) in porcine, equine and bovine muscle at levels ≥ 25 ppb.

3. Structures

THYREOSTATS	
	
2-thiouracil (TU) (MW = 128)	6-methyl-2-thiouracil (MTU) (MW = 142)
	
6-propyl-2-thiouracil (PrTU) (MW = 170)	2-mercapto-1-methylimidazole or Tapazole (TAP) (MW = 114)
	
6-phenyl-2-thiouracil (PhTU) (MW = 204)	2-mercaptobenzimidazole (MBI) (MW = 150)

B. EQUIPMENT

Note: Equivalent apparatus or instrumentation may be substituted unless specified for any of the following.

1. Apparatus

Note: Equivalent apparatus and instrumentation may be substituted for the following:

a. Robot Coupe® Processor - Robot Coupe U.S.A. Inc.

b. Centrifuge tubes - 50 mL, polypropylene tube, Falcon Cat. No. 352070, Becton Dickinson Labware.

c. Pipettors - 5 -100 µL, 100-1000 µL, Rainin EDP variable volume micropipettes.

d. Top-loading balance - PM 300, Mettler.

e. Analytical balance - Leco-250, Leco Corp.

f. Conical reaction vials - 5mL, Kontes Microflex, #749000-0005.

g. Microfilterfuge tubes - 0.45 µm Nylon 66, Cat. No. 7016-22, Rainin.

h. Evaporator - N-Evap, Organomation Associates.

i. Nitrogen source - Whatman N2-2010-(75-86).

j. Volumetric flasks - 1 L, 20 mL, 10 mL, and 1 mL.

k. Graduated cylinder - 1 L, 500 mL, 10 mL.

l. HPLC solvent filtering apparatus - with 0.45 µm filter.

m. Freezer - capable of attaining < -20 ºC.

n. Pipettes - 0.5 mL, 1 mL and 2 mL.

o. Centrifuges - Sorvall T6000B and VWR Model V.

p. Vortex mixer - Genie 2, Fisher Scientific.

q. Sonicating water bath - Aquasonic, Cat. No. 150T, VWR.

r. Glass centrifuge tubes - 15 mL, Cat. No. 8084, Pyrex.

s. Pasteur pipettes - disposable glass, 9 in. long.

t. HPLC vials and inserts.

u. SPE column - Silica Gel, Bond Elute, 0.5 g, 10 mL, Cat. No. 1211-3036, Varian.

2. Instrumentation

a. Micromass Quattro Micro equipped with electrospray LC interface coupled to a Waters 2695 HPLC and autosampler.

b. LC column - Phenomenex Prodigy 3 µm ODS(3) 100Å, 4.6 mm x 150 mm.

c. Guard column - Phenomenex ODS 4 mm x 3 mm.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted.

1. Reagents
   a. Acetonitrile (CH_3CN) - Cat. No. 015-4, Burdick & Jackson.
   b. Methylene chloride (CH_2Cl_2) - Cat. No. 9264-03, J. T. Baker.
   c. Methanol (MeOH) - Cat. No. 230-4, Burdick & Johnson.
   d. Sodium sulfate - Anhydrous, Cat. No. S421-1, Fisher Scientific.
   e. Formic Acid (HCO_2H) - Cat. No. 06440, Fluka.
   f. Water - HPLC grade or distilled de-ionized water.

2. Solutions

   Note: Unless otherwise noted, solutions may be stored at room temperature.

   a. 25% MeOH/CH_2Cl_2 (v/v):

      Mix 1 part MeOH with 3 parts CH_2Cl_2.

   b. HPLC mobile phases:

      i. A = 0.1% HCO_2H:

         Add 1 mL HCO_2H to a 1 L volumetric flask. Bring to volume with water (C.1.f).

      ii. B = 0.1% HCO_2H in 1:1 CH_3CN:MeOH:

         Mix 1 mL HCO_2H with 500 mL CH_3CN and 500 mL MeOH.

D. STANDARDS

1. Source

   Note: Equivalent sources for the standards can be used.

   a. 2-Thiouracil - Cat. No. 301507, Aldrich.
   b. 6-Methyl-2-thiouracil - Cat. No. 69400, Fluka.
   c. 6-Propyl-2-thiouracil - Cat. No. 82460, Fluka.
   d. 6-Phenyl-2-thiouracil - Cat. No. P3252, Sigma.
   e. 2-Mercapto-1-methylimidazole - Cat. No. 301507, Aldrich.
   f. 2-Mercaptobenzimidazole - Cat. No. M3205, Aldrich.

2. Preparation

a. Stock Standard Solutions (1 mg/mL):

Accurately weigh 20.0 ± 0.1 mg of each of the above standards into separate 20 mL volumetric flasks. Bring to volume with methanol. Store in separate vials at ≤ -10 °C.

b. Working Standard Solution (3 µg/mL):

Pipet 30 µL each stock standard to a 10 mL volumetric flask and bring to volume with methanol. Store at room temperature.

c. LC Standard Solution (25 ng/mL):

Pipet 10 µL working standard into LC vial and dilute with 400 µL methanol and 800 µL 0.1% formic acid. Prepare as needed and store at room temperature.

d. Storage and Stability: The stock standard solution is stable for 6 months when stored in a freezer at ≤ -10 °C.

E. SAMPLE PREPARATION

After removing excess fat from sample, homogenize with a food processor, transfer into plastic bags and store in a freezer at ≤ -10 °C. Let the sample thaw prior to analysis.

F. ANALYTICAL PROCEDURE

1. Extraction

a. Weigh 5 g homogenized tissue into 50 mL polypropylene centrifuge tube.

Note: At this time, weigh two 5 g portions of blank muscle tissue into 50 mL polypropylene centrifuge tubes. Use the first tube as a blank and fortify the second tube as a recovery by adding 42 µL of working standard (D.2.b) for a 25 ppb recovery.

b. Add 10 mL acetonitrile and cap.

c. Shake vigorously and vortex at least 1 minute until sample is dispersed.

d. Centrifuge at about 2500 rpm about 5 minutes.

e. Remove about 5 mL solution and place in 15 mL glass centrifuge tube.

f. Evaporate on N-Evap to dryness at ≤ 60 °C.

g. Add 0.5 mL methylene chloride to sample tube, cap and vortex briefly.

h. Sonicate at least 5 minutes.

2. SPE Column Cleanup

Note: Do not let the SPE column to go to dryness during steps F.2.b - F.2.f. Also for steps F.2.b - F.2.f allow liquid level to drain to top of column bed before adding next volume of liquid.

a. Add 1 g sodium sulfate to a silica gel SPE column, and position over waste container.

b. Wash column with 2 mL methylene chloride.

c. Add sample extract (F.1.h) to column.

d. Add 0.5 mL methylene chloride to sample tube, vortex and sonicate briefly, and add to column.

e. Add 2 mL 25% methanol/methylene chloride to sample tube and sonicate at least 1 minute.

f. Wash column with 2 mL methylene chloride.

g. Remove waste container and position a 5 mL reaction vial under column.

h. Elute column with 25% methanol/methylene chloride. Allow eluate to fully drain into tube.

Note: Irreversible adsorption of analytes to the SPE column may occur and may result in decreased recovery of some of the analytes. The use of a different solvent to elute the Thyreostat residues from the column may be necessary.

i. Evaporate to dryness on N-Evap at ≤ 60 °C.

j. Add 200 µL methanol and swirl gently to dissolve.

k. Add 400 µL 0.1% formic acid.

l. Cap and vortex briefly.

m. Add to 0.45 µm microfilterfuge tubes.

n. Centrifuge at about 8000 rpm until sufficient volume of filtrate has been collected for HPLC analysis.

o. Place sample in LC injection vial.

3. Instrument Operating Parameters - LC System

Note: The instrument parameters listed here are examples of one set of suggested optimization parameters. Others may yield equivalent results. The analyst should optimize parameters for the instrument used.

a. Set initial composition of mobile phase A to 93% and B to 7% at a flow rate of 0.5 mL/min. Allow system to equilibrate.

b. Injection volume: 20 µL

c. Elution gradient:

Time (min)	Flow Rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0	0.5	93	7
6	0.5	93	7
20	0.5	20	80

23	0.5	20	80
25	0.5	93	7
28	0.5	93	7

4. Mass Spectrometer Setup:

   Program the mass spectrometer to collect the product ions.

5. Instrumental Settings

   Note: Table contains recommended values. Instrumental settings may be adjusted, if necessary, to optimize performance.

   Typical LC/MS system setting:

   Polarity ES+

   Source Temperature 120 ºC

   Note: See Section K. 1. for additional settings.

6. Injection Sequence

   a. Inject external standard mixture and recovery.

   b. Inject the recovery and blank. Verify the absence of analyte carry over in the blank. If significant carry over is detected, inject solvent/ blank until reduced to acceptable level.

   c. Inject sample extract(s). Include additional washes and standards in the run as often as necessary to ensure proper identification of sample analytes.

   d. Reinject standard or recovery at the end of the run to verify instrument response.

7. Sample Chromatograms

   See Section K.2, Sample Chromatograms

G. DETECTION AND CONFIRMATION

1. For detection (screening):

   a. Plot ion chromatograms for each product ion monitored.

   b. Verify that all monitored product ions are present in the external standard and the positive controls.

c. Determine the retention times and abundances for a minimum of two product ions which includes the early eluter (TU) and the later eluting (PhTU) using the confirmation criteria for ion ratios.

Analyte	Product Ion Ratio	Parent mass	Retention Time examples
2-Thiouracil (TU)	84/112	129	5.32
2-Mercapto-1-methylimidazole (TAP)	88/56	115	6.93
6-Methyl-2-thiouracil (MTU)	126/84	143	7.61
6-Propyl-2-thiouracil (PrTU)	112/154	171	16.42
2-mercaptobenzimidazole (MBI)	118/93	151	17.09
6-Phenyl-2-thiouracil (PhTU)	188/146	205	18.63

2. For confirmation:

Calculate the ratios of product ions specified above for confirming testing.

a. Confirmation Criteria

Confirmation of thyreostat residues in a sample extract requires that the following criteria be met:

i. Retention time of the product ion peaks in the sample chromatograms must match that found in the external standard or recovery within ± 4%.

ii. At least 2 product ion peaks characteristic of the analyte are present with a signal to noise ratio of greater than 3.

iii. If two product ions are monitored, the presence of one sample ion ratio match that calculated for the external or recovery within a ± 10% arithmetic difference.

iv. The blank does not have confirmable analyte(s).

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment - Protective clothing, eyewear, gloves, and a hood where applicable.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
TU	May be harmful by inhalation, ingestion/skin absorption. Target organs Liver and Thyroid. May cause irritation to skin, mucus membranes and eyes.	Use a hood and wear protective clothing and gloves when handling standards.
MBI	Teratogen. May be harmful by inhalation, in contact with skin and if swallowed. Possible risk of impaired fertility and possible fetus risk.	See above
TAP	Possible Teratogen. May be harmful by inhalation, in contact with skin and if swallowed. Possible risk of impaired fertility and possible fetus risk.	See above
PhTU	May be harmful by inhalation, ingestion and skin absorption. Irritation to mucus membranes and upper respiratory tract.	See above
PrTU	Possible Carcinogen. May be harmful by inhalation, ingestion and skin absorption. May cause skin irritation. Irritation to mucus membranes and upper respiratory tract.	See above
MTU	Possible Carcinogen. May be harmful by inhalation, ingestion, and skin absorption. May cause skin irritation. Irritation to mucus membranes and upper respiratory tract.	See above
Acetonitrile/ methanol	Flammable, poisonous liquid	Wear protective clothing and gloves when handling acetonitrile and methanol.
Formic acid	Dangerously caustic to skin. Chronic absorption has been reported to cause albuminuria and hematuria.	Wear protective clothing and gloves when handling formic acid.

3. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Thyreostat standards as stated in above table.	See above.	Collect waste in a sealed container and store in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.
Acetonitrile/methanol	See above.	See above
Formic acid	See above.	See above

I. QUALITY ASSURANCE PLAN

1. Performance Standard
   a. No false positives from blank tissues.
   b. No false negatives at ≥ 25 ppb level fortification.

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	F.2.b-F.2.f. Silica gel column wash	Column must not go to dryness

3. Readiness To Perform
   a. Familiarization
      i. Phase I: Standards. Inject a mixed standard solution containing all six thyreostats at concentration equivalent to 25 ppb in sample extracts. Repeat analysis on three different days.
      ii. Phase II: Analyst fortified samples. Analyze one blank beef muscle tissue and beef muscle tissue fortified with 25 ppb mixed standards. Repeat the analyses two more days using blank pork muscle for day 2, and either blank pork or blank bovine muscle for day 3.

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

      iii. Phase III: Check samples for analyst accreditation.

(a) 6 samples fortified at 25 ppb level of each analyte. Any combination of species may be used, and set must include 1 blank for a total of 7 samples.

(b) Report analytical findings to Supervisor and QAM.

(c) Letter from QAM is required to commence official analysis.

b. Acceptability criteria.

Refer to I. 1.

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. Matrices: Bovine, porcine or equine muscles.

b. Sample receipt, minimum weight: approximately 500 g.

c. Condition upon receipt: chilled or frozen.

d. Sample storage:

i. Condition: frozen (≤ -10 ºC) for blended/homogenized samples.

6. Sample Set

Each set must include the following:

a. Blank muscle (negative control).

b. Muscle recovery (positive control).

c. Samples.

7. Sensitivity

a. Minimum proficiency level (MPL): 25 ppb.

J. WORKSHEET

The worksheets on the following pages are an example.

Thyroestat Analysis by LC-MS-MS

TST 2.____

Analyst: _____

Date Started: _____

Date Completed: _____

Reagents	ILN	ILN of Equipment
Standard		
Acetonitrile (CH_3CN)		
Methylene Chloride (CH_2CL_2)		
SPE column		n/a
Sodium Sulfate (Na_2SO_4)		
25% Methanol/Methylene Chlorine ($MeOH/CH_2CL_2$)		
Methanol (MeOH)		
Formic Acid (HCO_2H)		
Mobile Phase A (0.1% HCO_2H in H_2O)		LC/MS/MS
Mobile Phase B (0.1% HCO_2H in CH_3CN:MeOH)		LC/MS/MS

Equipment	ILN	
Balance		Cln __
TurboVap		Temp____
Centrifuge(s)		
LC/MS/MS		

	Sample ILN	Form #	Tissue Type	Sample Weight (g) 5.0 ± 0.5 g	Comments
1	Tissue Blank				QC Tissue ILN:_____
2	Rec____				spiked w/ ____ul using pipette ILN_____
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					
21					
22					
23					
24					
25					
26					
27					
28					
29					
30					

★ Internal Check spiked by: _____ using pipette ILN:_____; unknown to analyst at time of analysis.

Thyreostats Worksheet

Method:

Analyst
Date Began
Date Ended

Sample	TU parent=129						TAP parent=115						MTU parent= 143					
	Ret Time	Product Ions 112	Product Ions 84	Ion Ratio	Std Confirm?	Rec Confirm?	Ret Time	Product Ions 88	Product Ions 56.7	Ion Ratio	Std Confirm?	Rec Confirm?	Ret Time	Product Ions 126	Product Ions 84	Ion Ratio	Std Confirm?	Rec Confirm?
Standard					na	na					na	na					na	na
Recovery					na	na					na	na					na	na

Sample	PrTU Parent=171						MBI Parent=151						PhTU Parent =205					
	Ret Time	Product Ions 154	Product Ions 112	Ion Ratio	Std Confirm?	Rec Confirm?	Ret Time	Product Ions 118	Product Ions 93	Ion Ratio	Std Confirm?	Rec Confirm?	Ret Time	Product Ions 188	Product Ions 146	Ion Ratio	Std Confirm?	Rec Confirm?
Standard					na	na					na	na					na	na
Recovery					na	na					na	na					na	na

	Standard Ranges			
	Ret Time		Ion Ratio	
	Low	High	Low	High
TU				
TAP				
MTU				
PrTU				
MBI				
PhTU				

	Recovery Ranges			
	Ret Time		Ion Ratio	
	Low	High	Low	High
TU				
TAP				
MTU				
PrTU				
MBI				
PhTU				

K. APPENDIX

1. Additional Instrument settings:

Calibration static	2
Capillary voltage (kV)	3.3
Cone voltage	30
Extractor voltage	3.0
RF Lens voltage	0.20
Desolvation Temperature	450 ºC
Cone Gas Flow (L/H)	41
Desolvation Gas Flow	700
LM 1 Resolution	14.5
HM 1 Resolution	14.5
Ion Energy	0.2
Entrance	5
Collision	20
Exit	1
LM 2 Resolution	15.0
HM 2 Resolution	15.0
Ion Energy	1.5
Multiplier voltage	700
Gas Cell Pirani Pressure (mbar)	5.98e - 3

2. Mass Spectra

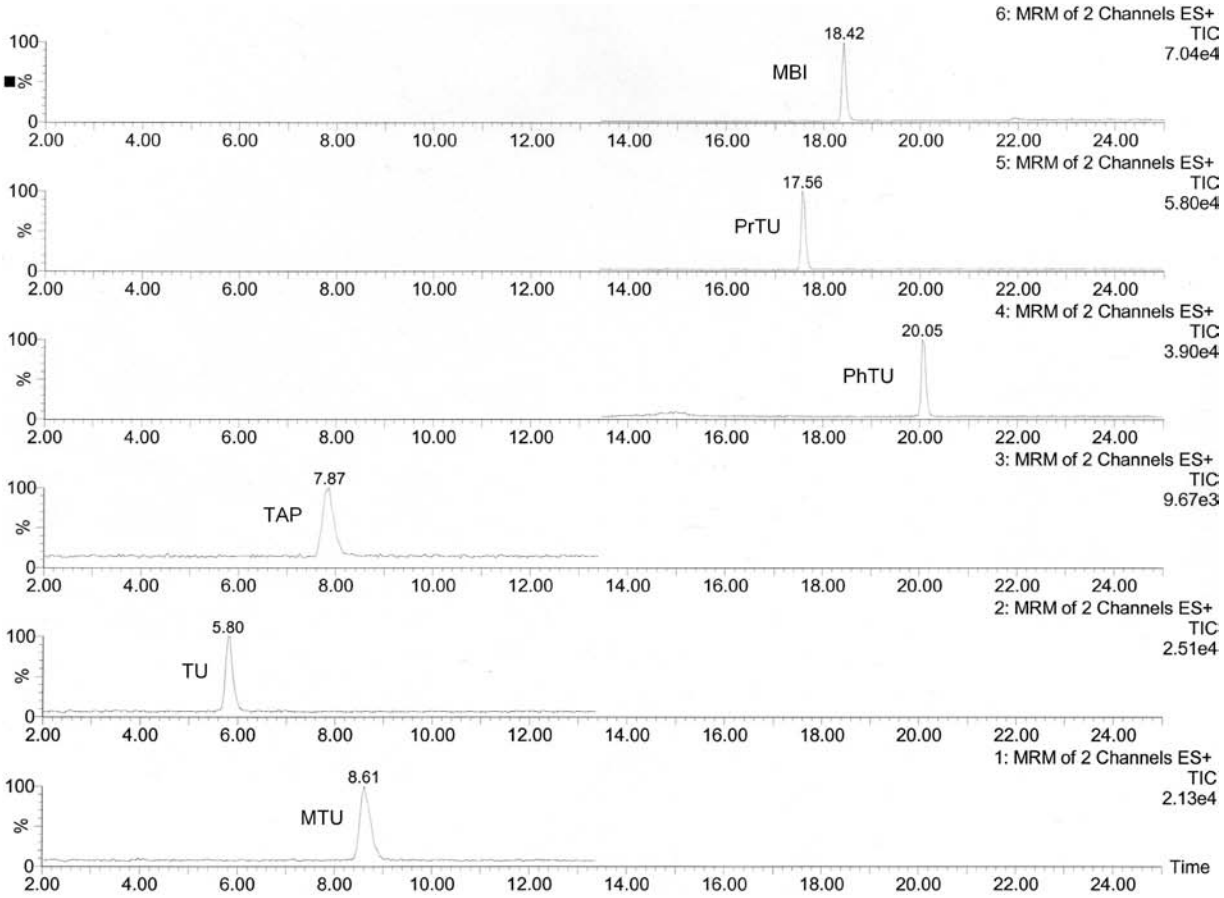


Figure 1. Spectra of 25 ppb Thyreostats External Standard

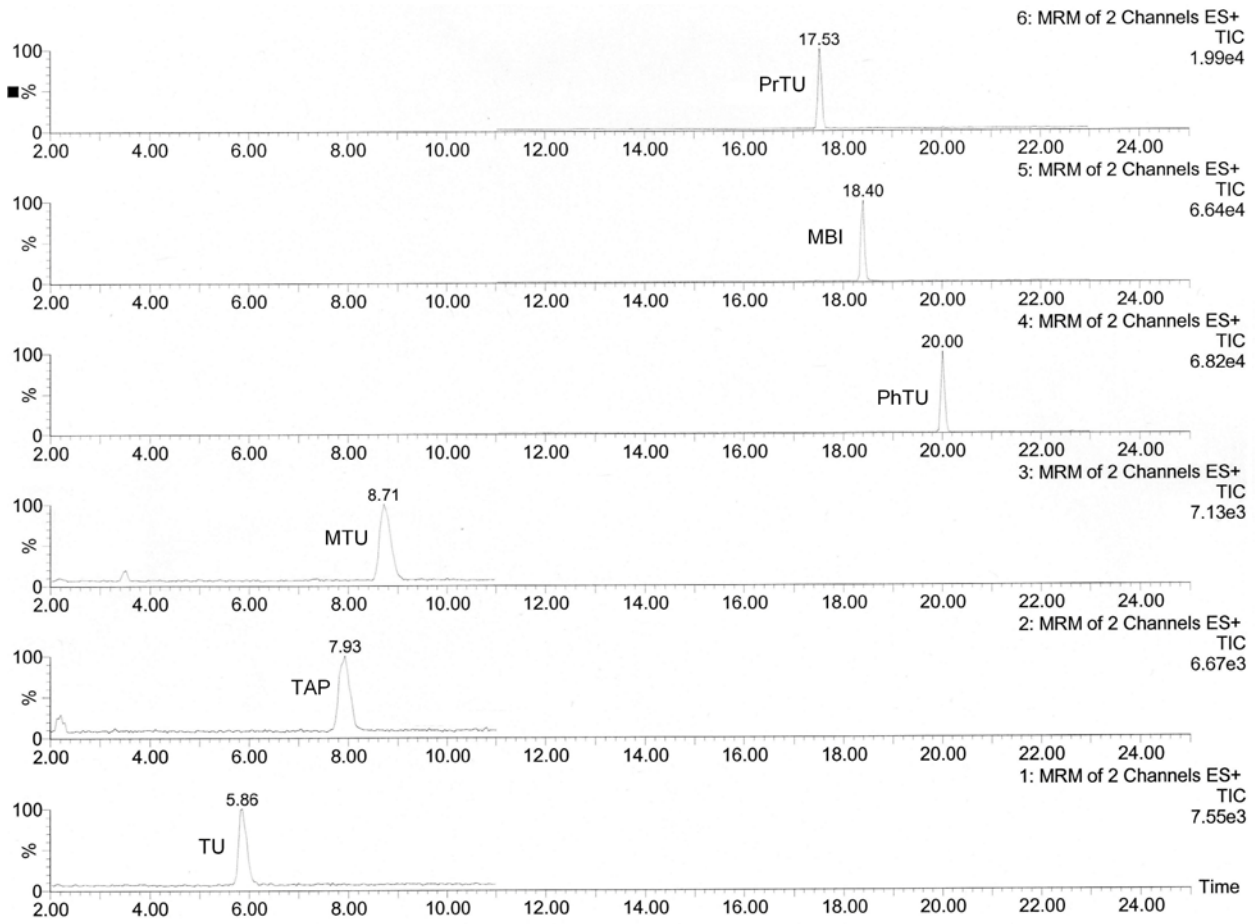


Figure 2. Spectra of 25 ppb Thyreostats Beef Muscle Recovery

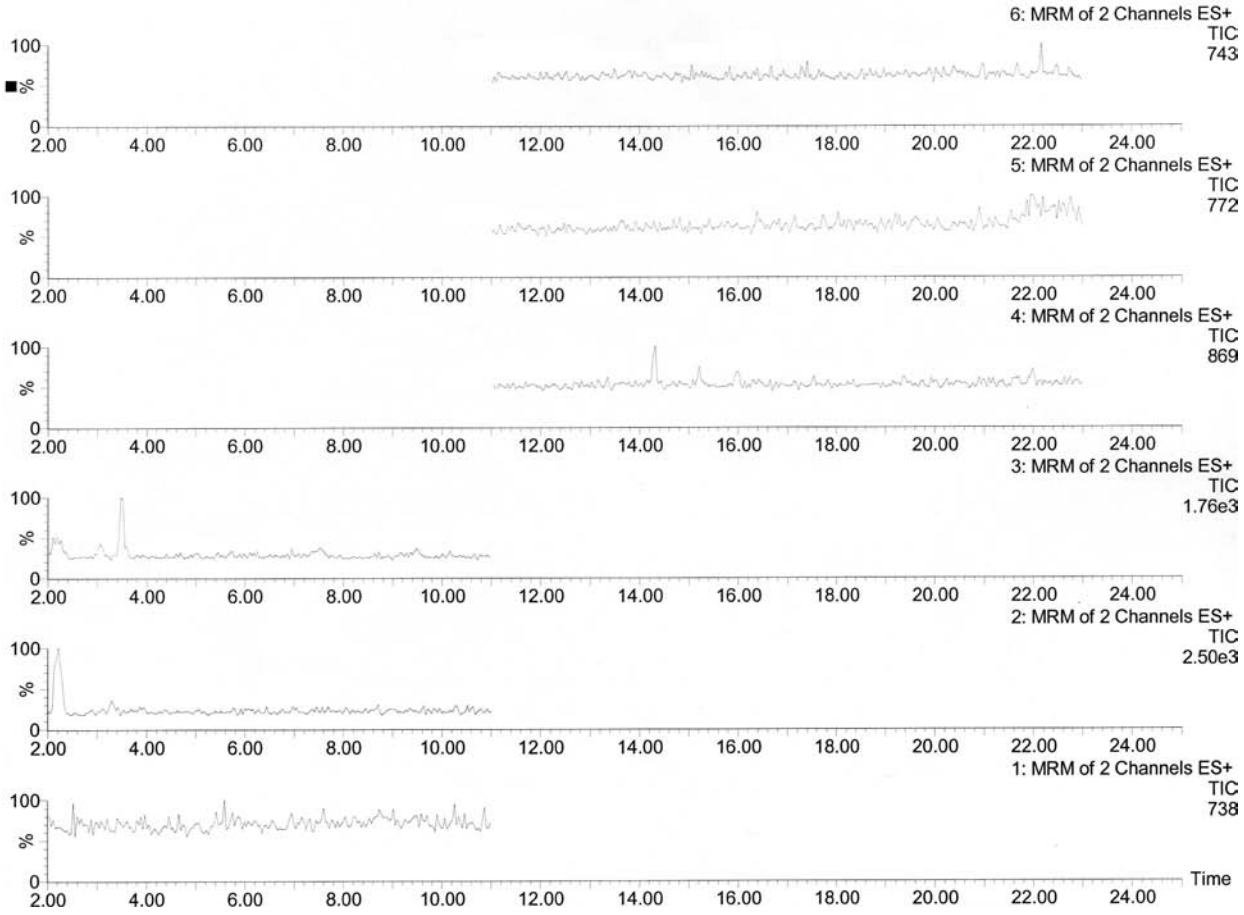


Figure 3. Spectra of Blank Beef Muscle

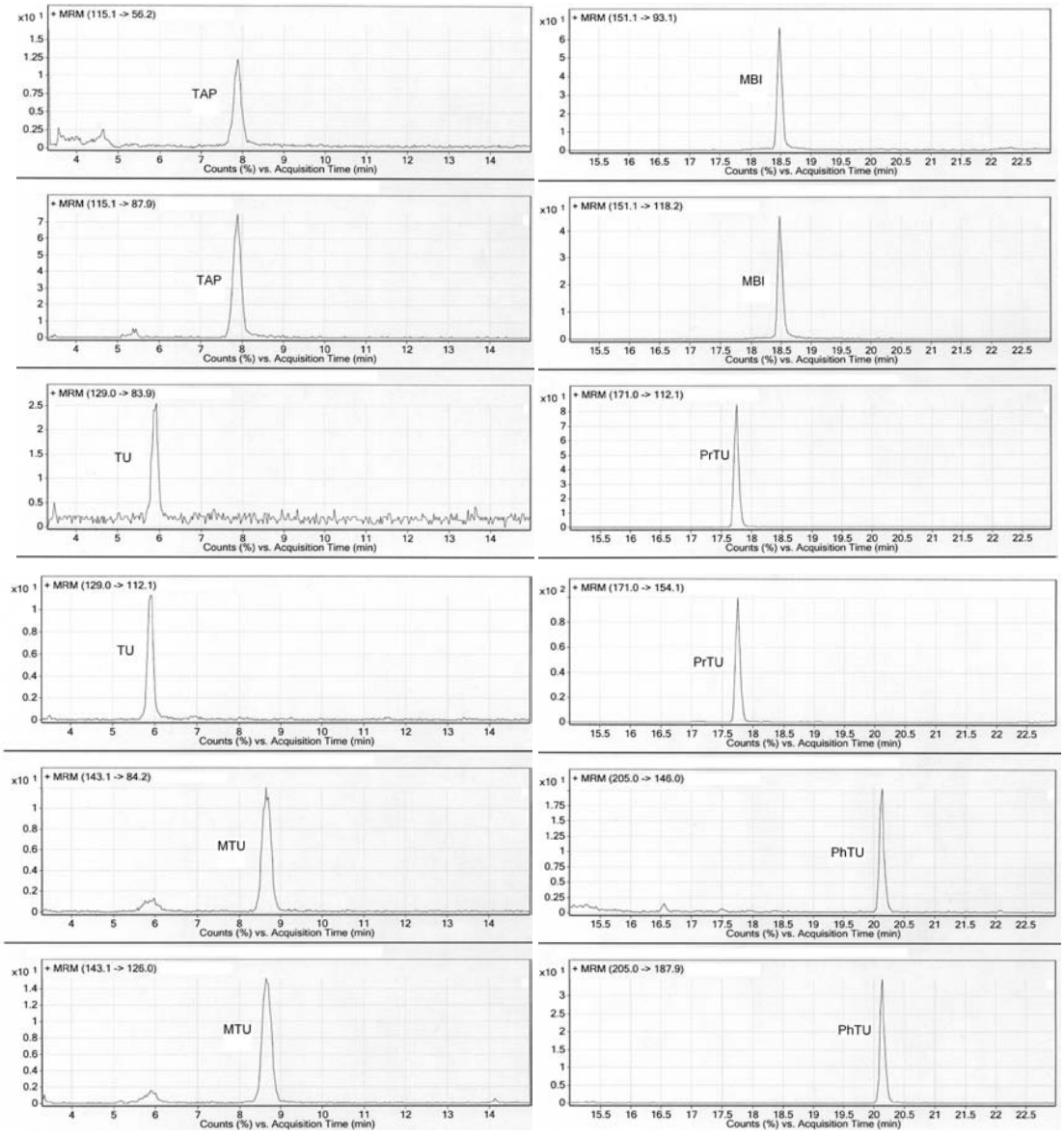


Figure 4. Spectra of 25 ppb Thyreostats Pork Muscle Recovery

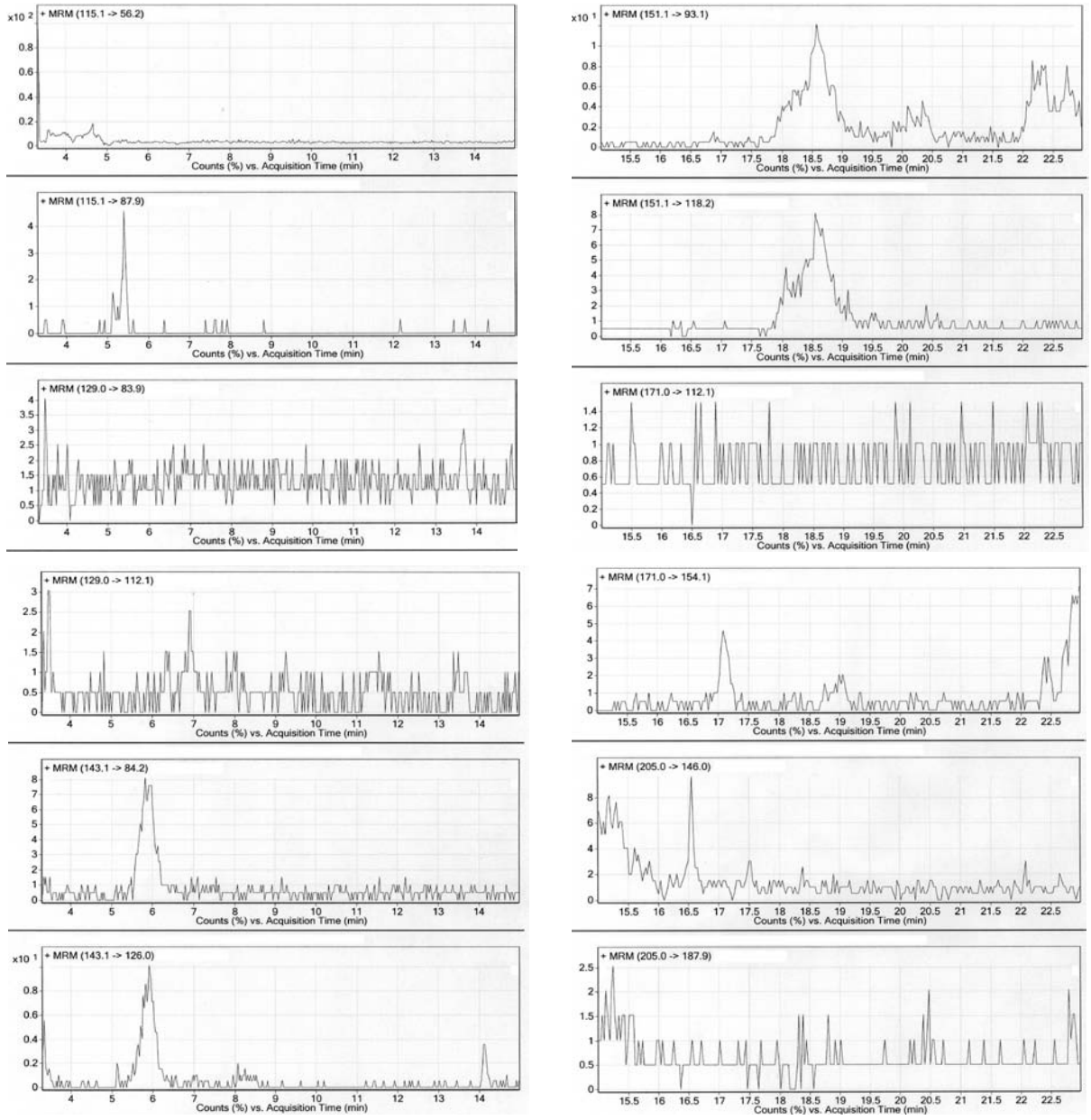
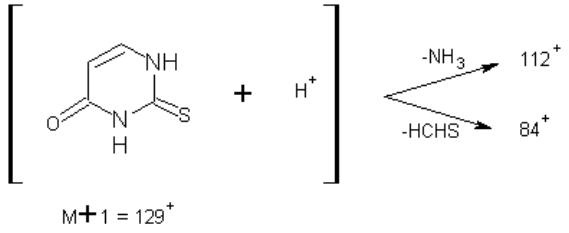
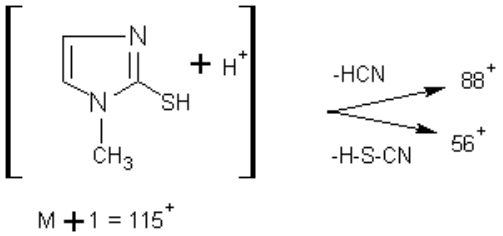
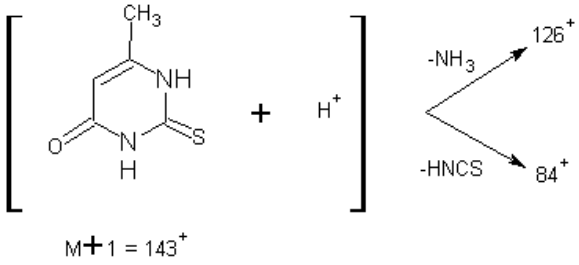
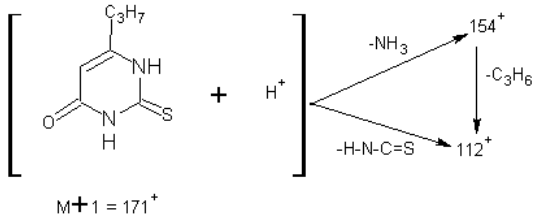
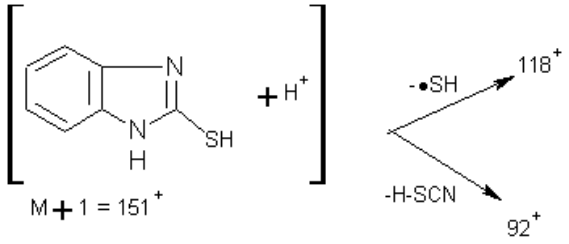
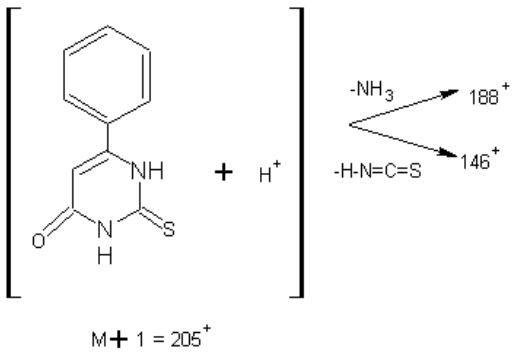


Figure 5. Spectra of Blank Pork Muscle

3. Possible Fragmentation Patterns of Thyreostats Product Ions

2-thiouracil (TU) (MW = 128)	2-mercapto-1-methylimidazole or tapazole (TAP) (MW = 114)
	
6-methyl-2-thiouracil (MTU) (MW = 142)	**6-propyl-2-thiouracil (PrTU) (MW = 170)**
	
2-mercaptobenzimidazole (MBI) (MW = 150)	**6-phenyl-2-thiouracil (PhTU) (MW = 204)**
	

4. Reference

Steven J. Lehotay, Alan R. Lightfield, Nichelangelo Anastassiades, and David J. Smith. "Simultaneous Analysis of Beta-Agonists and Thyreostats in Animal Tissues by LC/MS-MS and in-line Fluorescence". 4th International Symposium on Hormone and Veterinary Drug Residue Analysis, Antwerp, Belgium, June 4-7, 2002.

L. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Division.