HALOFUGINONE

Contents

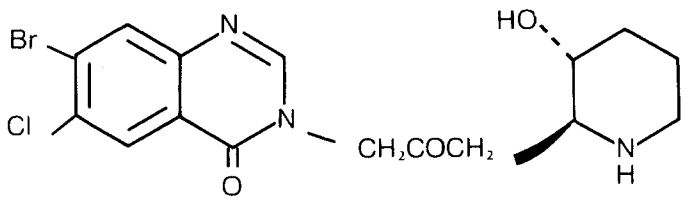
I. DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory and Structure

a. Halofuginone is a coccidiostat for young chickens and young turkeys. In higher doses, halofuginone is a growth depressant, impairs feed utilization, and reduces feed intake. In rats it causes alopecia. The compound is prohibited from use during the last four days before slaughter (withdrawal period).

b. Halofuginone is extracted from enzyme-digested chicken liver as a free base into ethyl acetate and partitioned into ammonium acetate buffer solution. The halofuginone is concentrated with Sep-Pak C_{18} cartridges and eluted with methyl alcohol. The eluant is evaporated to dryness and the residue dissolved in the HPLC mobile phase. Analysis is achieved by HPLC using a BONDAPAK C_{18} column and a variable wavelength UV detector.



HALOFUGINONE

2. Applicability

This method is applicable for chicken and turkey liver.

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Centrifuge, IEC, or equivalent, fitted with a rotating head capable of accepting 200 mL centrifuge bottle.

b. Food processor.

c. pH meter, Horizon Model 5998-10, or equivalent.

d. Single pan balance, Mettler P-1200, or equivalent.

e. Analytical balance, Mettler Gram-atic Type 6, or equivalent.

f. Water bath, Thelco Model 83, or equivalent.

g. Vortex mixer, Matheson Scientific Super-Mixer, or equivalent.

h. Evaporator, rotary.

i. General laboratory equipment and silanized glassware.

NOTE: Silanize the halofuginone-collecting glassware, the 15 mL test tubes, and the 250 mL round-bottom flasks.

2. Instrumentation

a. High-pressure liquid chromatograph system, Waters, or equivalent.

i. Waters Model 6000A solvent delivery system.

ii. Waters Intelligent Sample Processor (WISP), Model 710B.

iii. Kratos Spectroflow 773 variable wavelength dectector.

iv. Waters Data Module Model 730 integration system.

b. HPLC column, Waters μ-BONDAPAK C_{18}, 30 mm $\times$ 4 mm id, or equivalent.

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solutions List

a. Ethyl acetate, Burdick & Jackson, distilled in glass, or equivalent.

b. Water, Milli-Q®, or equivalent.

c. Acetonitrile, Burdick & Jackson, distilled in glass, or equivalent.

d. Methanol, Burdick & Jackson, distilled in glass, or equivalent.

e. AquaSil, Pierce, water-soluble silanizing fluid.

f. Sodium chloride, Fisher Scientific analytical reagent, or equivalent.

g. Sodium carbonate, Fisher Scientific analytical reagent, or equivalent.

h. Sodium carbonate solution (10% w/v). Dissolve sodium carbonate (100 g) in water and dilute to 1 L in a volumetric flask.

i. Salt-saturated sodium carbonate solution (5% w/v). Dissolve sodium carbonate (50 g) in water and dilute to about 750 mL in a 1 L volumetric flask. Add sodium chloride, mixing, until the solution is saturated. Bring to volume.

j. Glacial acetic acid, Fisher Scientific, reagent grade, or equivalent.

k. Ammonium acetate, HPLC grade.

l. Ammonium acetate buffer (0.25M; pH 4.3). Add ammonium acetate (19.27 g) and acetic acid (30 mL) in water to 1 L volumetric flask. Dilute to volume with water.

m. Ammonium acetate buffer (0.125M; pH 4.3). Dilute 500 mL of 0.25M ammonium acetate buffer to 1 L with water in a volumetric flask.

n. Trypsin, Fisher Scientific, or equivalent.

o. Filter paper, Whatman GF/F, or equivalent.

p. Filter units, Millex®, HV 0.45 μm filter unit.

q. Sep-Pak C_{18} cartridge.

s. Preparation of Sep-Pak C_{18} cartridge.

   i. In preparation of HPLC analysis, wash cartridges sequentially with 2 mL methanol and 5 mL water.

   ii. In preparation for MS analysis, wash cartridges sequentially with 5 mL methanol and 10 mL of water.

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

Halofuginone hydrobromide (halofuginone).

Hoechst-Roussel Agri-Vet Company
Manager, Nutritional Research
P.O. Box 2500
Somerville, NJ 08876-1258
Telephone: 205/231-2000

2. Preparation of Standards

a. Stock standard.

Weigh 50 mg of halofuginone into a 250 mL volumetric flask. Dilute to volume with 0.25M ammonium acetate buffer (200 μg/mL).

b. HPLC calibration standards.

mL Used from Stock Standard	*Final Volume (mL)*	*Concentration (ppm)**
0.0	100	0
0.25	100	0.05
0.50	100	0.10
1.0	100	0.20

*Concentration calculated based on 20 g of sample.

c. Fortification standard.

Pipet 1 mL of stock standard into a 100 mL volumetric flask and dilute to volume with distilled water (2 μg/mL).

3. Storage Conditions

All standards are to be refrigerated at 4° C in tight glass bottles.

4. Shelf Life Stability

a. Stock standards: Replace every three months.

b. HPLC calibration standard: Replace every three months.

c. Fortification standard: Replace every month.

NOTE: Stability is determined based on the storage conditions in section D.3.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Extraction

a. Homogenize the liver sample and blank tissue using a food processor.

b. Weigh 20 g ± 0.1 g of the homogenized liver into a 200 mL centrifuge bottle.

c. Perform a 0.1 ppm recovery by adding 1 mL of fortification standard solution to 20 g of blank tissue.

d. Add 10 mL of water and 500 mg trypsin.

e. Adjust to pH no less than 8 nor more than 8.5 by addition of 10% w/v sodium carbonate solution.

f. Incubate in 40° C water bath for 3 hours. (This is a convenient stopping point. Samples may be refrigerated and stored overnight or weekend.)

g. Cool to room temperature. (Warm, if refrigerated.)

h. Add 10 mL 10% w/v sodium carbonate solution and mix.

i. Add 100 mL ethyl acetate and macerate for 3 min with the tissuemizer.

j. Centrifuge for 2 min at 10° C, 2000 rpm.

k. Decant the ethyl acetate into a 500 mL separatory funnel via a vacuum suction apparatus. Clean the decanting tube between each sample with ethyl acetate.

l. Repeat steps i-k adding the second volume of ethyl acetate to the first extract.

m. Add 50 mL salt-saturated sodium carbonate solution (5% w/v) and shake vigorously for 1 min.

n. Discard the aqueous layer.

NOTE: If a precipitate forms in the aqueous layer, filter the layer plus precipitate into a beaker through a layer of glass wool contained in a funnel. Use ethyl acetate to wash precipitate and return the cleaned aqueous/ethyl acetate layers to the separatory funnel. The aqueous layer can then be drained off and discarded.

o. Add 50 mL 0.125M ammonium acetate buffer and shake for 1 min.

p. Transfer the ammonium acetate layer (lower layer) into a 250 mL separatory funnel.

q. Repeat steps o and p, adding the second ammonium acetate extract to the first extract.

r. Discard the ethyl acetate layer.

s. Add 10 mL ethyl acetate to the ammonium acetate buffer and wash gently for 10 sec.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

t. Transfer the ammonium acetate layer (lower layer) into a 250 mL round-bottom flask and evaporate all remaining ethyl acetate from the buffer solution. This takes approximately 20-30 min on the rotary evaporator. There must be no ethyl acetate odor remaining.

u. Transfer into a 100 mL volumetric flask and bring to volume with 0.125M ammonium acetate buffer solution.

v. Filter through a Whatman GF/F filter paper, discarding the first few mL of filtrate.

w. Elute 20 mL of filtrate through a pre-washed Sep-Pak C_{18} cartridge. Discard filtrate. (Refer to section C, Reagent and Solution List, item s.i, for prewash instructions.) At this point, sample cartridge may be refrigerated overnight or over a weekend before proceding to next step.

x. Wash the cartridge with 3 mL of water and discard the washing.

y. Elute halofuginone from the cartridge with 5 mL methanol into a 15 mL conical centrifuge tube.

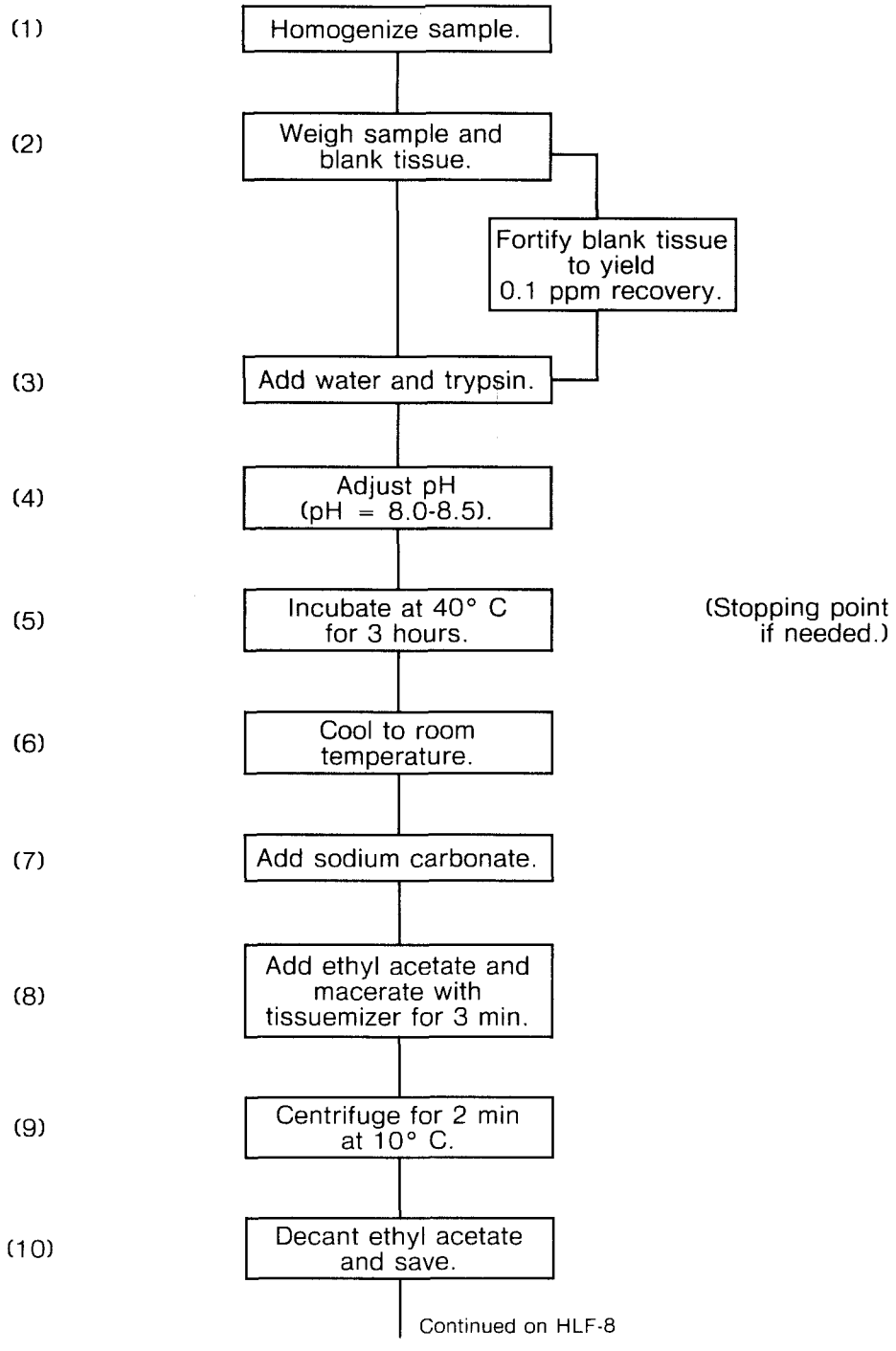
z. Under a gentle stream of nitrogen, evaporate to 1 or 2 mL. Pass through a Millex® HV 0.45 filter unit. Rinse the unit with a small amount of methanol and continue to evaporate sample to dryness.

aa. Bring the residue to 400 μL with mobile phase, vortex, and transfer to WISP micro vial for HPLC analysis.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

2. Flow Chart Summary



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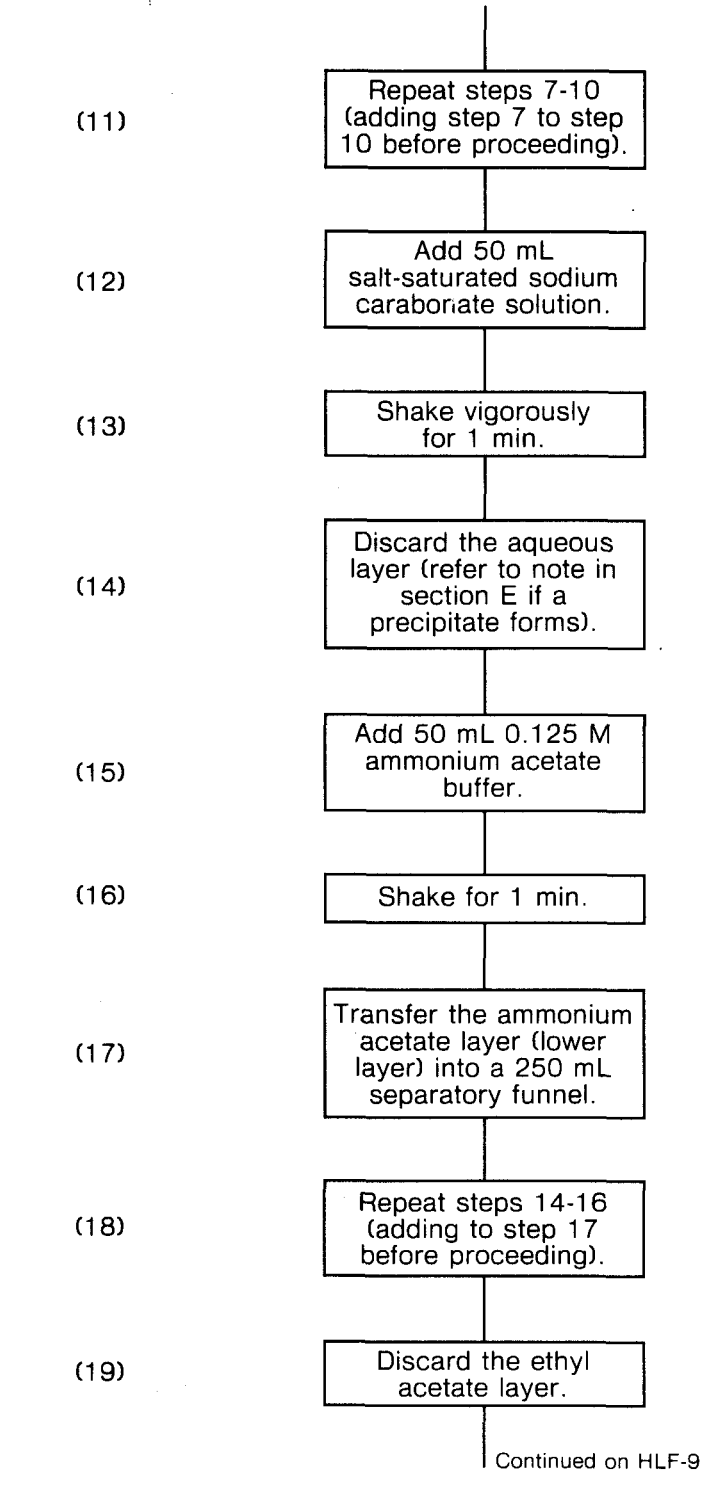
I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)



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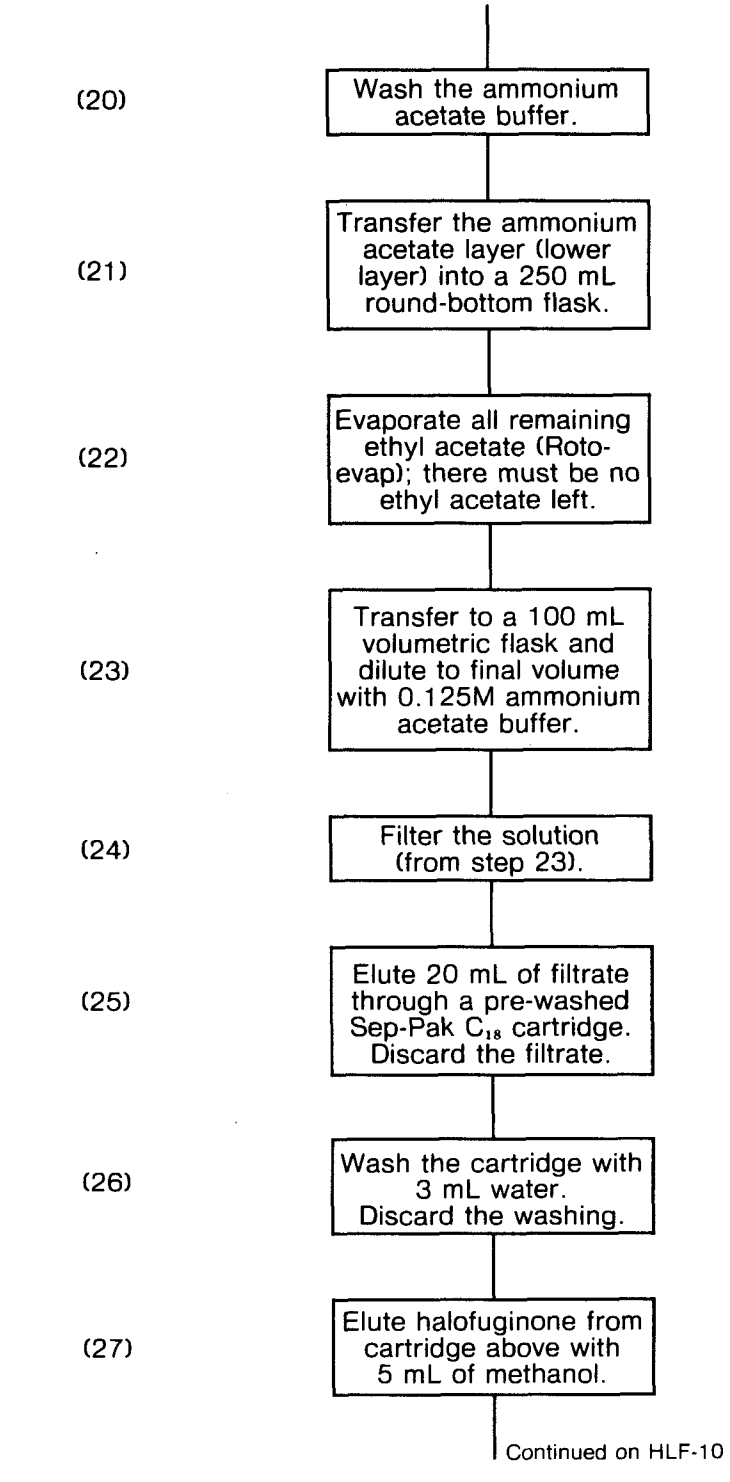
I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)



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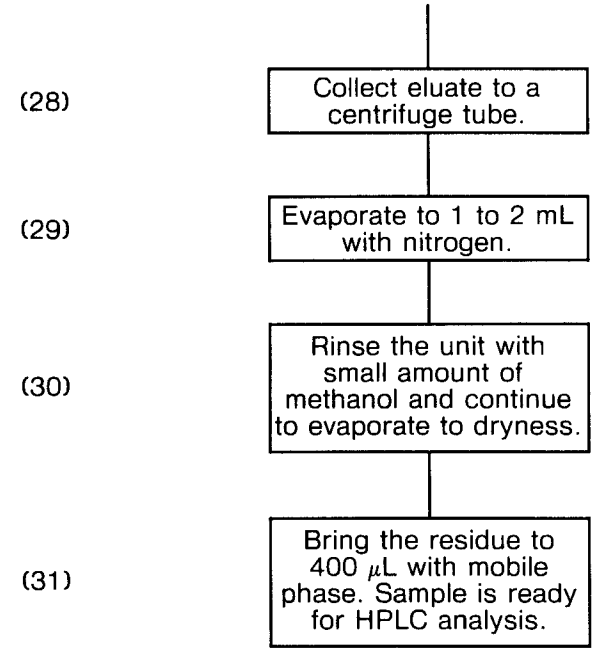
I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

NOTE: Equivalent instrumentation may be substituted.

a. Wavelength: 243 nm.

b. Column: μ-BONDAPAK C_{18}, 30 cm × mm id, 100 × 2.1 mm C-8 HP #79916 MD microbore.

c. Mobile phase: Acetonitrile/0.25M ammonium acetate buffer/water 5:3:12 adjusted to pH 4.3 with acetic acid.

d. Flow rate: 0.125 mL/min.

e. Oven temperature: 40° C.

f. Pressure: 2,300-2,800 PSI.

g. Injection volume: 25 μL.

h. Retention time: 5-7 min.

i. Run time: 10 min.

j. Sensitivity: 0.02 AUFS.

k. Chart speed: 0.25 cm/min.

NOTE: Parameters may be changed to optimize chromatography.

2. Interferences

No known interferences.

I. DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

The integrator calculates the nanograms of halofuginone in the sample. The ppm is calculated by the following formula.

$$\frac{\text{initial vol}}{\text{wt of sample}} \times \frac{\text{final vol}}{\text{aliquot}} \times \frac{\mu\text{g detected}}{\text{inj vol}}$$

i.e.,

$$\text{ppm halofuginone} = \frac{100\ \text{mL}}{20\ \text{g}} \times \frac{400\ \mu\text{L}}{20\ \text{mL}} \times \frac{\mu\text{g detected}}{50\ \mu\text{L}} = 2 \times \mu\text{g detected}$$

2. References

a. American Hoechst Corporation—NADA 130-951.

b. Hazleton Biotechnologies Corporation—Analytical Method 32 (1982).

c. Hazleton Biotechnologies Corporation—Analytical Method 98 (1984).

d. Oneida Research Services, Inc., "Confirmation Assay for Halfuginone in Chicken Liver Using Negative Ion Chemical Ionization Mass Spectrometry/Mass Spectrometry".

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the following page, *Halofuginone*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

WORKSHEET: HALOFUGINONE

Analyst: _____

Date: _____

Recovery of This Set: _____

Running Average of 10 Latest Acceptable Recovery Values: _____

COV of Running Average: _____

Standard Curve Data

$y = mx + b$

b = _____

m = _____

r = _____

F = _____

SAMPLE IDENTIFICATION	CALCULATION CONTROL POINTS					SAMPLE DATA			COMMENTS
	Sample Wt gm	Initial vol mL	Aliquot mL	Final Vol µL	Inject vol µL	Peak Height cm	µg halofug	ppm halofug*	
1. 0 µg Standard					50.0	0.0			
2. 0.025 µg Standard (1/2x)									
3. 0.05 µg Standard (1x)									
4. 0.10 µg Standard (2x)									
5. 0.1 ppm Recovery	20.0	100.0	20.0	400.0					
6. R926-									
7.									
8.									
9.									
10.									
11.									
12.									
13.									
14.									
15.									
16.									
17.									
18.									
19.									

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

Element	*Analytical Range (ppm)*	*Acceptable Recovery (%)*	*Repeatability %CV*
Halofuginone	> 0.05	60-115	≤ 20

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	Mobile phase pH.	4.3 ± 0.05.
b.	Glassware.	Must be silanized.
c.	Trypsin addition.	500 mg ± 50 mg.
d.	pH	8.25 ± 0.25.
e.	Maceration time.	3-4 min.
f.	Time constraint.	Steps i-t of section E must be completed in < 1 hr.
g.	Ethyl acetate removal and bath temperature.	37° C ± 1° C; no odor remaining.
h.	Filtrate volume for elution.	20 mL ± 0.02 mL.
i.	Elution volume of methanol.	5 mL ± 0.01 mL.
j.	Evaporation temperature.	< 40° C.
k.	Final volume.	400 µL ± 10 µL.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—4 levels, 3 replicates each, on 3 separate days.

(a) Blank.

(b) 12.5 ng in 50 µL injection.

(c) 25 ng in 50 µL injection.

(d) 50 ng in 50 µL injection.

ii. Phase II: Fortified Samples—4 levels, 3 replicates each over a minimum of three days.

(a) Blank.

(b) 0.05 ppm.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

(c) 0.10 ppm.

(d) 0.20 ppm.

NOTE: Phases I and II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) 14 samples from FSIS Western Laboratory (samples submitted by supervisor if only one laboratory is performing this test).

(b) Report analytical findings to Chemistry Division.

Notification from Chemistry Division required to commence official analysis.

b. Acceptability criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: 1 per week per analyst, or 20% of official samples analyzed (whichever is smaller).

ii. Blind samples (requires "dummy" forms).

iii. Records to be maintained by analyst and reviewed by supervisor and Laboratory QA Officer.

(a) All replicate findings.

(b) Running average difference between replicates.

(c) All % recoveries.

(d) Running average, standard deviation, and CV for recoveries.

(e) Appropriate CUSUM charts.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

5. Sample Acceptability and Stability	a. Matrix: Chicken liver. b. Sample receipt size: Varied; enough to obtain matrix for all required screening, quantitation, and confirmation tests. c. Condition upon receipt: Frozen. d. Sample storage: i. Time: 6 months. ii. Condition: Frozen.
6. Sample Set	a. Tissue blank. b. Fortified blank tissue at 0.10 ppm. c. Samples.
7. Sensitivity	a. Lowest detectable level (LDL): 0.05 ppm. b. Lowest reliable quantitation (LRQ): 0.05 ppm. c. Minimum proficiency level (MPL): 0.05 ppm.

II. CONFIRMATORY METHOD

Procedure for MS/MS Confirmation of Halofuginone Violations

a. Take 60 mL of the remaining filtrate from Determinative Method, section E, step w, and pass through a prewashed Sep-Pak C_{18} cartridge. Discard the filtrate coming through the cartridge.

b. Wash the cartridge with 3 mL of water and discard the washing. Be sure all water is removed before proceding to next step.

c. Elute the halofuginone from the cartridge with 5 mL of methanol and collect the methanol in a 15 mL conical centrifuge tube.

d. Evaporate to dryness under a gentle stream of nitrogen at 40° C.

e. Add 240 μL of mobile phase to the centrifuge tube and vortex thoroughly to dissolve the haofuginone residue. Pass through a 0.45 micron filter. Sample is ready for HPLC analysis at this point.

f. Prepare standards such that the peak height of the sample is bracketed by a standard of lower height and a standard of higher height. Use a 200 μL injection.

g. Chromatograph and collect the fraction of eluant containing the halofuginone peak. Also chromatograph the 0.1 ppm recovery and collect the halofuginone fraction.

h. Remove the acetonitrile from the eluate by rotary evaporation at 40° C.

i. Pass the sample through a prewashed Sep-Pak C_{18} cartridge and discard washing.

j. Wash the cartridge with 5 mL of water and discard washing.

k. Elute halofuginone from the cartridge into a 15 mL test tube with 5 mL methanol. Collect the eluate and evaporate to dryness under a gentle stream of nitrogen.

l. Send the sample, the 0.1 ppm recovery, and a 1 μg standard to:

Dr. James Sphon HHF 423
Division of Contaminants Chemistry
200 C Street, FOB #8
Washington, DC 20204