NICARBAZIN IN POULTRY TISSUES

Contents

I. Determinative Method

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 9
G. Calculations 14
H. Hazard Analysis 15
I. Worksheet 16
J. Quality Assurance Plan 19

II. Confirmatory Method

A. Introduction 21
B. Equipment 21
C. Reagents and Solutions 21
D. Standards 21
E. Sample Preparation and Cleanup 22
F. Analytical Confirmation Procedure 22
G. [Reserved]
H. Hazard Analysis 24
I. Worksheet 24
J. Quality Assurance Plan 27

I. DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Nicarbazin is a 1:1 molar mixture of HDP and DNC. HDP is 4,6-Dimethyl-2-pyrimidinol and DNC is 4,4-Dinitrocarbanilide. Nicarbazin is used in chickens to prevent coccidiosis, an infectious disease caused by intestinal protozoan parasites.

Nicarbazin is extracted from chicken tissue with ethyl acetate. After filtration and evaporation of the extract, the residue is purified by liquid-liquid partitioning and alumina column chromatography. Analytical separation and measurement are accomplished by high performance liquid chromatography (HPLC) with UV detection of the DNC portion of the nicarbazin complex at 340 nm.

2. Applicability

This method is applicable for chicken muscle and liver.

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

NOTE: Equivalent apparatus may be substituted for that specified if necessary.

a. Platform shaker, Eberbach Model 6010.

b. Cieria homogenizer, Sorvall Omni-Mixer, Type OM. NOTE: A Tissuemizer or equivalent *cannot* be used.

c. Virtis flask (100 mL).

d. Du Pont homogenizing cups, part no. 17047.

e. Rinco rotary vacuum evaporator with temperature-controlled water bath.

f. Waters Sep-Pak alumina B cartridges, part no. 51820.

g. Filter paper, Schleicher and Schuell 588.

h. 50 mL polypropylene tubes, Falcon 2098.

i. Waring blender, with 1 quart jar.

j. Hobart food chopper, equipped with 5/32″ hole plate.

2. Instrumentation

HPLC system consisting of the following components, or equivalent:

a. Waters Model M-6000 high pressure pump.

b. Waters Wisp Model 710B autosampler and autoinjector.

c. Waters Model 450 UV-visible detector, set at 340 nm.

d. Chromatographic data system, HP 1000, integrator, or chart recorder.

e. IBM C_{18} column, 4.6 × 100 mm, 3 μm particles.

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

1. Reagent List

a. Ethyl acetate, HPLC grade.

b. Hexane, HPLC grade.

c. Acetonitrile, HPLC grade.

d. Dimethylformamide (DMF), HPLC grade.

e. Methanol, HPLC grade.

f. Water, deionized using Millipore system.

2. Solution List

Methanol/water, 3: 1 (v/v).

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

Eli Lilly and Company
Indianapolis, IN 46285

NOTE: Each new lot of standard reference material should be prepared as stated below and compared to the current standard.

NOTE: Information as to the exact ratio of DNC to HDP for that lot should be provided and will be needed for calculations.

2. Preparation of Standards

a. Stock standard (1000 μg/mL): Weigh 100 mg of nicarbazin reference standard. Transfer to a 100 mL volumetric flask and dissolve in 75 mL of dimethylformamide (DMF) by heating at 75-80° C. Cool, dilute to volume with DMF, and mix well.

b. Working standard (20 μg/mL): Pipet 2.0 mL of stock standard solution into a clean 100 mL volumetric flask. Dilute to volume with ethyl acetate.

c. HPLC standards:

i. 6 μg/mL spiking solution. Pipet 3.0 mL of working standard into a 10 mL volumetric flask and dilute to volume with methanol.

ii. 4 μg/mL spiking solution. Pipet 2.0 mL of working standard into a 10 mL volumetric flask and dilute to volume with methanol.

iii. 2 μg/mL spiking solution. Pipet 1.0 mL of working standard into a 10 mL volumetric flask and dilute to volume with methanol.

3. Storage Conditions

All standard solutions should be stored at room temperature.

4. Shelf Life Stability

a. Stock standard—stable for one month.

b. Working standard—stable for one month.

c. HPLC standard—stable for three days.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

a. Liver. Blend approximately 1 lb of tissue in a 1-qt Waring blender jar until a consistent homogenate is obtained.

b. Muscle. Pass approximately 1 lb of tissue through a Hobart food chopper equipped with a 5/32″ hole plate. Mix thoroughly by hand-kneading to produce a uniform homogenate.

NOTE: *Do not* homogenize tissues completely. A Tissuemizer or equivalent *cannot* be used since the homogenate produces interferences in recovery of analyte and filters slowly as a consequence of high efficiency blending.

2. Sample Extraction

a. If using:

   i. Eberbach platform shaker: Weigh 10.0 g of a representative sample of ground or minced tissue into a 50 mL polypropylene centrifuge tube.

   ii. Virtis homogenizer: Weigh 10.0 g of a representative sample of ground or minced tissue into a 100 mL Virtis flask.

b. Prepare a recovery sample at 2 ppm by fortifying a 10.0 g sample of untreated control tissue with 1.0 mL of working standard. Include an untreated control tissue sample with each set of samples.

c. If using:

   i. Eberbach platform shaker: Add 25 mL of ethyl acetate to the sample and shake by hand vigorously for 10 sec. Place on the platform shaker and shake for 3 min at high speed. Centrifuge the sample at 2000 rpm for 2 min.

   ii. Virtis homogenizer: Add 25 mL of ethyl acetate to the sample and homogenize at low speed for 3 min.

d. Being careful to avoid transferring tissue, decant the ethyl acetate supernatant from either step c.i or c.ii into a 250 mL round-bottom flask or other suitable container.

   NOTE: When blending sample in a Virtis homogenizer, decant supernatant through a filter paper. Any tissue that is decanted with the supernatant should be scraped off the filter paper and placed back into the Virtis flask.

e. Repeat steps c.i to d or c.ii to d twice more, using the same filter paper and flask to collect the extract from each sample. After the three extracts have been collected, rinse the filter paper with three 2 mL portions of ethyl acetate.

f. Evaporate the ethyl acetate using a rotary vacuum evaporator with a temperature-controlled water bath at 45-50° C.

   NOTE: For liver and muscle tissue, there will be about 1-2 mL of oil left in the residue.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

g. Add 50 mL of hexane to the round-bottom flask and quantitatively transfer to a 125 mL separatory funnel.

h. Rinse the round-bottom flask with 10 mL acetonitrile and transfer to the separatory funnel. Shake the separatory funnel vigorously for approximately 1 min. Let the separatory funnel sit until the phases are sufficiently separated.

i. Carefully drain the acetonitrile layer (lower phase) into the same round-bottom flask previously used, ensuring that all of the acetonitrile layer is recovered.

j. Re-extract the hexane phase with two 10 mL portions of acetonitrile and add these acetonitrile extracts to the same round-bottom flask for each sample. Discard the hexane phase.

k. Evaporate the acetonitrile extract completely to dryness with a rotary vacuum evaporator. Reconstitute the dried extract by adding 2 mL of dimethylformamide to the flask.

l. Attach a 10 mL syringe barrel or similar apparatus to the alumina B cartridge as a solvent reservoir. Rinse the alumina B cartridge with 4 mL of DMF. *Do not allow the column to go dry at this step.*

NOTE: All elution steps are performed as an open column procedure without vacuum. It may be necessary to apply gentle pressure with a rubber bulb or other similar device to initiate flow.

m. Quantitatively transfer the solubilized sample to the alumina B cartridge by rinsing the sample flask with two 2 mL portions of DMF and by adding these rinses to the cartridge. Discard eluate.

n. Wash the cartridge with one 5 mL portion of DMF and discard eluate. It is important to eliminate any DMF with pressure from a rubber bulb.

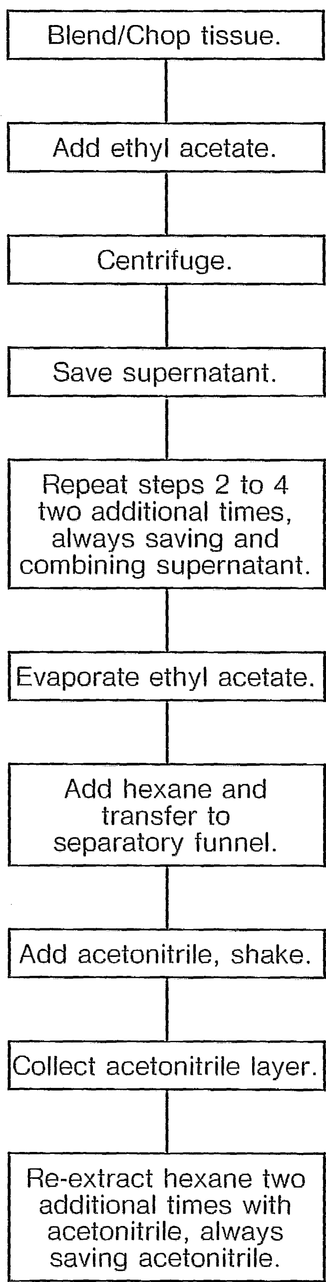
o. Elute the compound of interest into a 10 mL volumetric flask by adding 10 mL of methanol to the cartridge. A rubber bulb can be used to increase the flow rate to approximately 2 mL/min and to drain all of the DNC from the column. Adjust final volume in volumetric flask to mark, using methanol.

p. Inject 10 μL of sample into HPLC for quantitation.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

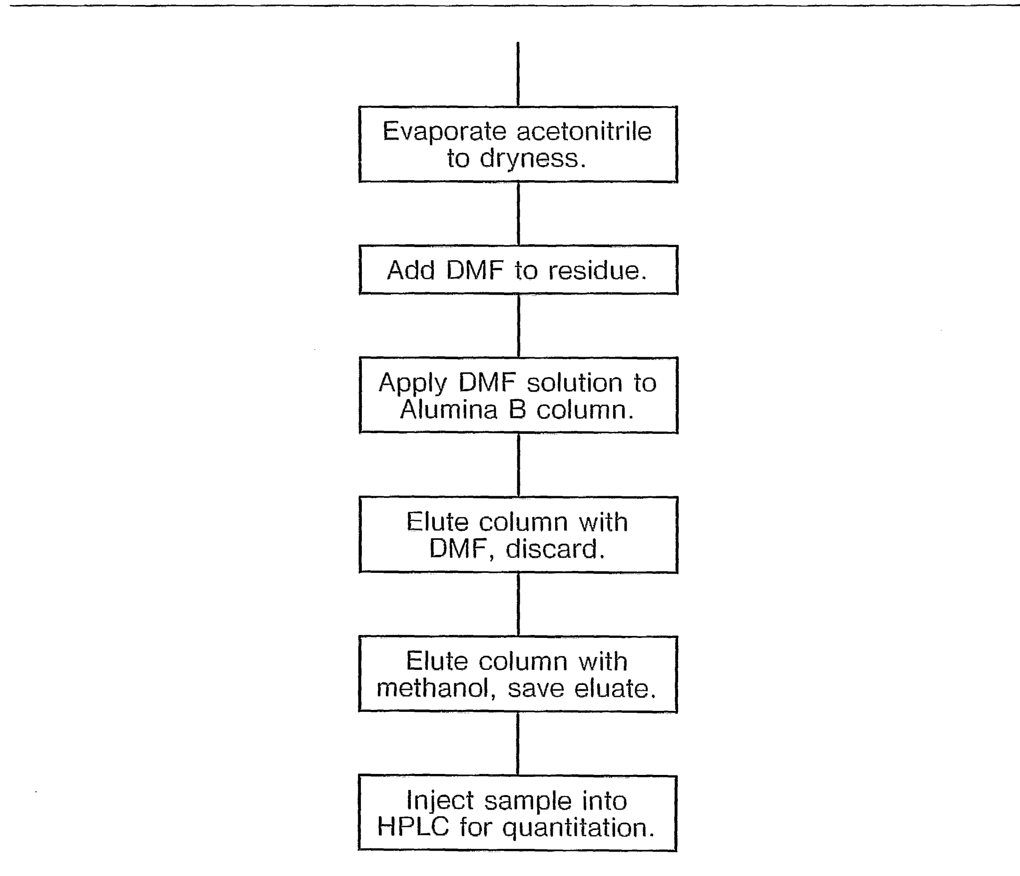
3. Flow Chart Summary



Continued on NIC-8

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

a. Guard column	C_{18}, 3 μm particles (optional)
b. Analytical column	IBM C_{18} column, 4.6 × 150 mm, 3 μm particles.
c. Mobile phase	Methanol/Water 75/25 (v/v).
d. Flow rate	0.8 mL/min.
e. Injection volume	10 μL.
f. Column temperature	40° C.
g. Retention time	Approximately 5-6 min.
h. Detector setting	UV 340 nm (0.1 AUFS for samples $\leq$ 0.5 ppm, and 0.2 AUFS for samples $>$ 0.5 ppm).

2. General Operation

a. The baseline should be stable before beginning a run. Reference standard responses should be sufficient for reliable measurement ($>$ 2 × signal to noise ratio). DNC should be separated from impurities in the sample. The retention time of DNC should not vary more than 20 sec in a particular run.

b. Record the daily operating conditions on the chromatograms and measure the peak area of DNC. If necessary, dilute sample extract with mobile phase to yield peak responses within the range of the standard curve.

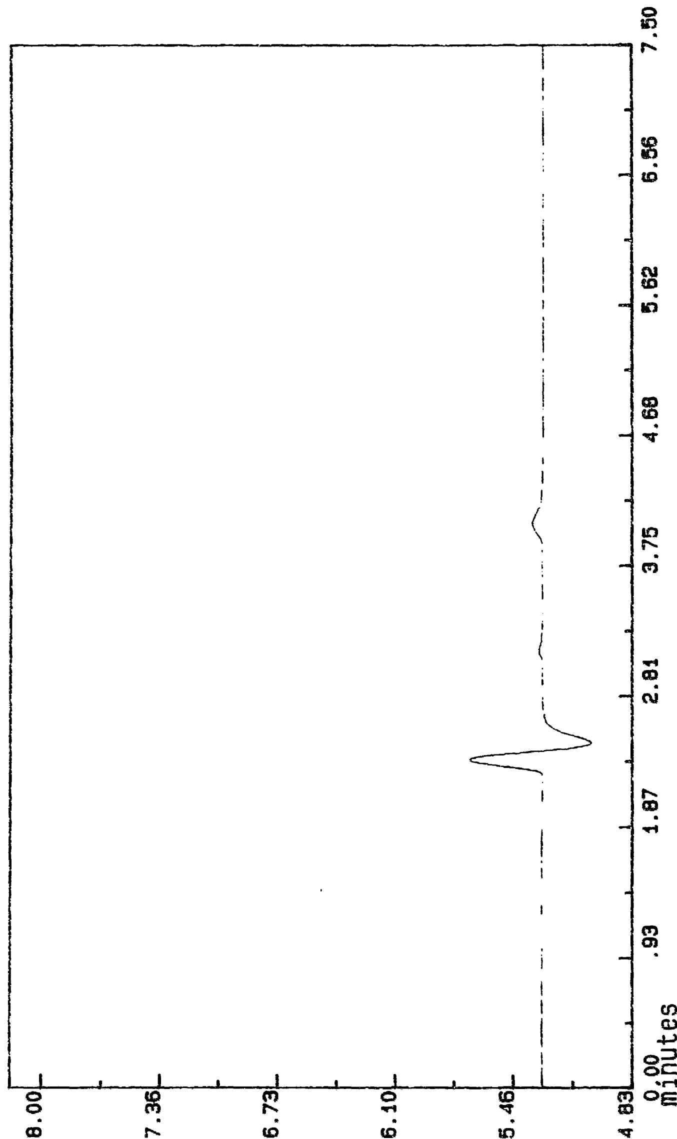
3. Interferences

None known.

I. DETERMINATIVE METHOD

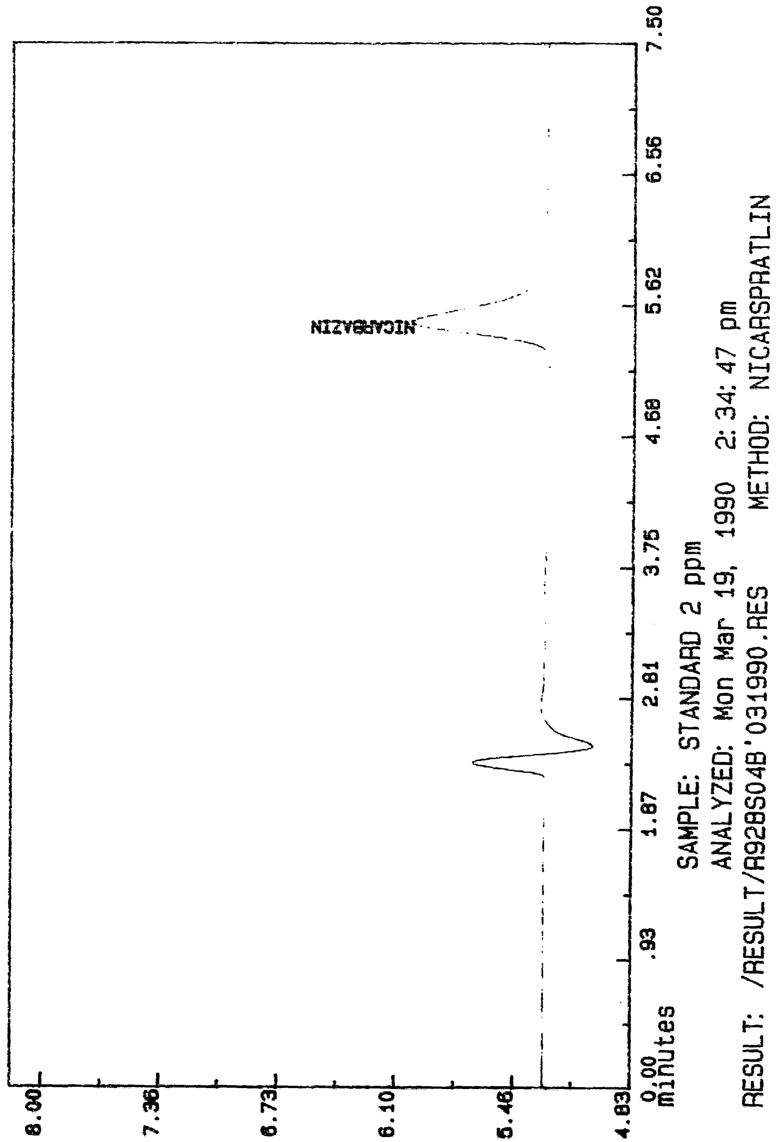
F. ANALYTICAL QUANTITATION (Continued)

4. Sample Chromatograms



I. DETERMINATIVE METHOD

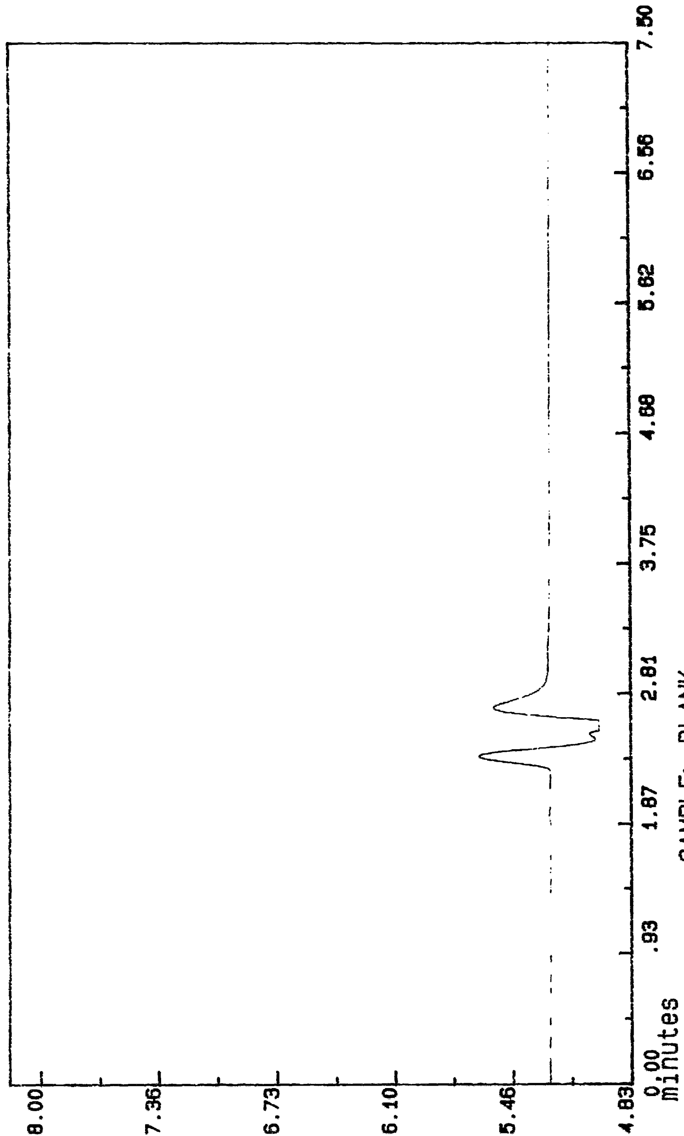
F. ANALYTICAL QUANTITATION (Continued)



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

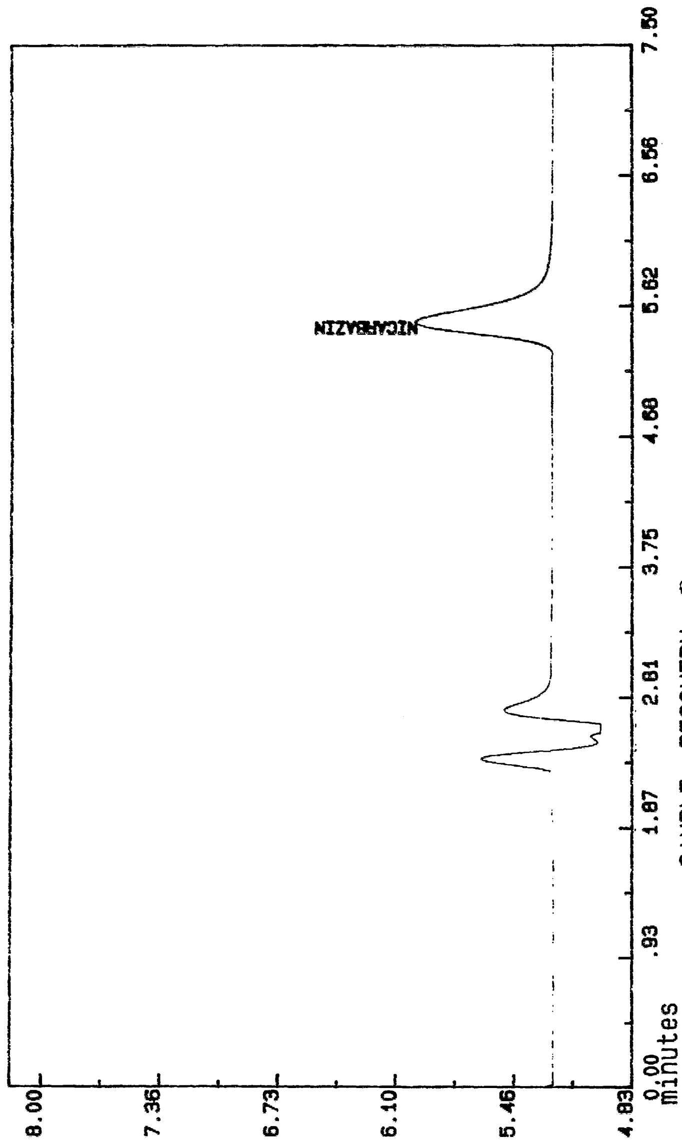
AMPLITUDE/1000
Force Normalized
(5.00, 8.00)



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

AMPLITUDE/1000
Force Normalized
(5.00, 8.00)



I. DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

a. By an acceptable means, measure the peak area of the 0, 2, 4, and 6 µg/mL HPLC standards (section D.2.c). Using the peak area and associated µg/mL values, construct a standard calibration curve by least squares computation, as indicated in the Chemistry Quality Assurance Handbook, Volume I, 1.5.67.

b. According to the following equation, calculate percent recovery (R_i) in the 2 µg/mL fortified control tissue.

$$R_i = \frac{C_r \times V \times AF \times 100}{\mu g \text{ fortified}}$$

where:

C_r = concentration of nicarbazin recovery sample as determined from the standard curve µg/mL.

V = final volume in mL (normally 10 mL).

AF = aliquot factor (normally 1.0).

c. Determination of the concentration (ppm) of nicarbazin tissue samples is accomplished by comparison with the standard peak areas and adjustment for the mean percent recovery of the fortified untreated control samples, if less than 100%, as shown below.

$$\text{ppm nicarbazin} = \frac{C_s \times V \times AF \times Fdnc \times 100}{W \times R_a \times 0.7089}$$

where:

W = weight (g) of tissue extracted.

R_a = last 10 running average percent recovery.

C_s = concentration of nicarbazin sample as determined from standard curve (µg/mL).

Fdnc = actual fraction of DNC in the nicarbazin reference standard (0.674 to 0.730).

0.7089 = theoretical fraction of DNC in the nicarbazin reference standard.

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Analysis of Nicarbazin in Poultry Tissues		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.		
3. Procedure Steps		*Hazards*	*Recommended Safe Procedures*
	C. Reagents		
	Acetonitrile Hexane Ethyl acetate Methanol DMF	Flammable. Vapors are irritating and may be absorbed through the skin, producing toxic effects.	Work in an efficient fume hood. Use plastic gloves when adding solvent and shaking the funnels.
4. Disposal Procedures	Organic solvents	(see above)	Store in the nonchlorinated solvent waste container until disposed of by contractor or in-house specialist.

I. DETERMINATIVE METHOD

I. WORKSHEET

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

WORKSHEET FOR NICARBAZIN ANALYSIS

Analyst:_______________ Analyst Code: [ ]

Date: _______________

CONDITION 15 cm x 4.6 mm ID Zorbax
ODS–C–18 Column
Mobile Phase: 25% H_2O in CH_3OH (V/V)
Column temperature: 40°C
Flow: 0.8 ml/min (usual pressure 1000 psi, 500 – 2500 psi acceptable)
Injection volume 10 μl

NOTE: Evaporation Step – DO NOT ALLOW SAMPLE TO GO TO DRYNESS

SAMPLE IDENTIFICATION:	CRITICAL CONTROL POINTS

PROCEDURE KEY:	Sample Weight Grams	Spiking Volume ml	Shaking Time in Min	Final Volume ml	Peak Hght or Area	PPM found or % Recovery	Comments
STANDARD 0 PPM							
STANDARD 2 PPM							
STANDARD 4 PPM							
STANDARD 6 PPM							
BLANK	10.0 gms		3 min	10 ml			
RECOVERY 2 PPM		2 ml					
1 Sample #							
2 Sample #							
3 Sample #							
4 Sample #							
5 Sample #							
6 Sample #							
7 Sample #							
8 Sample #							
9 Sample #							
10 Sample #							
11 Sample #							
12 Sample #							
13 Sample #							
14 Sample #							
15 Sample #	V		V	V			

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Peformance Standards

Compound	*Analytical Range (ppm)*	*Acceptable Recovery (%)*	*Repeatbility %CV*	*Reproducibility %CV*
Nicarbazin†	0-6.0 ppm	80-110	10	15‡

† DNC component.
‡ Reproducibility is estimated value; actual value to be determined after validation study.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample weight	10.0 ± 0.1 g
b. Volume of DMF	2.0 ± 0.1 mL
c. Volume of residual solution	10.0 ± 0.2 mL

3. Readiness To Perform

a. Familiarization.

i. Phase I: Duplicate sets of standard curves on each of three days at 0, 2.0, 4.0, and 6.0 ppm.

ii. Phase II: Duplicate self-fortified recovery samples using chicken liver tissue spiked at 0, 2.0, 4.0, and 6.0 on three days.

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

iii. Phase III: Check samples for analyst accreditation. Twelve chicken liver tissue samples, ranging from 0.4 to 6 ppm, including blanks, submitted by supervisor or Laboratory QA Officer.

b. Acceptability criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least one check sample biweekly per analyst.

ii. Random replicates or blind samples may be chosen by the supervisor or Laboratory QA Officer.

iii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) CUSUM charts.

(c) All recovery values.

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

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

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 if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrix: Liver.

b. Sample receipt size: Sufficient for all quantitative analyses and sample reserved for confirmation (at least 500 g).

c. Condition upon receipt: Chilled or frozen.

d. Sample storage:

i. Time: Not determined; stability study required.

ii. Condition: Not determined; stability study required.

6. Sample Set

a. Blank.

b. Fortification at 2 ppm.

c. Samples.

7. Sensitivity

a. Lowest detectable level (LDL): 0.1 ppm.

b. Lowest reliable quantitation (LRQ): 0.4 ppm.

II. CONFIRMATORY METHOD

A. INTRODUCTION

1. Theory

Extracts from the determinative method are separated from interferences by reverse phase gradient HPLC. A diode array detector is used to determine the UV spectra of the suspected nicarbazin peak, which are compared to those of a standard.

2. Applicability

This method is applicable to all tissues referenced in the determinative method, section A.2, NIC-1.

B. EQUIPMENT

1. Apparatus

Refer to determinative method, section B.1, NIC-2.

2. Instrumentation

a. Liquid chromatograph: Hewlett-Packard Model 1090M, equipped with autoinjector, binary gradient option, and diode array detector (DAD), controlled by a Hewlett-Packard Model 79994 HPLC Chemstation.

b. Analytical column: 25 cm × 4.6 mm id, containing 5 μm Econosphere C_{18} spherical particles (Alltech Associates).

NOTE: Equivalent instrumentation may be substituted for that specified if necessary.

C. REAGENTS AND SOLUTIONS

Refer to determinative method, section C, NIC-3.

D. STANDARDS

Refer to determinative method, section D, NIC-4.

II. CONFIRMATORY METHOD

E. SAMPLE PREPARATION AND CLEANUP

Refer to determinative method, section E, NIC-5 through NIC-8.

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

a. HPLC parameters.

i. Flow rate: 1.0 mL/min.

ii. Injection volume: 25 μL.

iii. HPLC gradient.

NOTE: Since the system varies depending upon the matrix, it is recommended that the gradient system be used only if interfering peaks were observed when running the analysis with the isocratic system. If the isocratic system is the choice, use 75:25 methanol/water as the eluent. Otherwise the following conditions could be used as guidelines for a gradient system.

(a) Initial mixture: 60:40 methanol/water.

(b) Initial hold: 7 minutes.

(c) Gradient 1: Ramp to 80:20 in 4 minutes.

(d) Hold 1: 5 minutes.

(e) Gradient 2: Ramp to 60:40 in 4 minutes.

(f) Hold 2: 3 minutes.

b. DAD parameters.

i. Signal A: 345 ± 2 nm (4 nm bandwith).

ii. Signal B: 223 ± 2 nm.

iii. Signal C: 258 ± 2 nm.

iv. Signal D: 313 ± 2 nm.

v. Signal E: 373 ± 2 nm.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

vi. Reference signal for signals A-E: 550 ± 50 nm.

vii. Spectrum range: 210-450 nm.

viii. Store spectrum: Peak controlled, threshhold 1 mAU.

ix. Stop time: 16 min (peak elutes at 14 min).

c. Instructions for data acquisition.

Set up instrument operating parameters as described above. Allow the DAD to warm up at least 30 min before attempting analysis. After sample run is complete, use Chemstation software to integrate chromatographic peaks generated by signals A-E and produce a report of the results. In addition, a background-corrected spectrum of the peak of interest should be generated and printed out to allow a visual comparison of spectra.

2. Required Samples

A confirmation set consists of an external standard, a recovery, a tissue blank, and the presumptive positive sample from the determinative method. The external standard and recovery should be prepared at concentrations similar (±50%) to those found in the sample.

3. Criteria for Confirmation

a. Retention time specificiation. Retention time of the sample peak should match that of the standard and recovery within a 1% tolerance.

b. Wavelength ratio specification. All ratios of signals B-E to A for the sample must match those of the standard or recovery within 10%.

Absorbance Ratios for Nicarbazin

*Wavelength**	*Absorbance (Normalized)*
345	1.00
225	0.58
260	0.070
315	0.53
375	0.48

*Specified wavelength ± 2 nm.

c. Spectral comparison specification (visual confirmation). The background-corrected spectrum of the sample peak must match those of the standard or recovery with respect to location of absorbance minima and maxima. In addition, the overall shape of the sample spectrum must match that of the standard or recovery.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

4. Interferences

Some extracts from the determinative procedure may contain a number of compounds that can interefere in the absorbance range of 220-260 nm when isocratic (75:25 methanol:water) conditions are used for eluting. The gradient used has proven adequate for separating these interferences when using the specified column. Substitution of a different column may require development of a different gradient for adequate separation.

H. HAZARD ANALYSIS

Refer to Determinative Method, section H.

I. WORKSHEET

The worksheet on the facing page, *Nicarbazin HPLC-DAD Confirmation Data Form,* can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

NICARBAZIN HPLC-DAD CONFIRMATION DATA FORM

Sample Number: ______________________ **Tissue:** ______________________

Analyst: ______________________ **Date Run:** ______________________

Sample	Ret. Time	Peak ht/area 345	225	260	315	375
Blank						
Standard						
Recovery						

Pk ht/Area Ratio (relative to 345)					
	225	260	315	375	Spectrum shape OK?
Blank					
Standard					
Recovery					

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

a. No false positives at 4 ppm.

b. No false negatives at 4 ppm.

2. Critical Control Points and Specifications

NOTE: Sample cleanup and preparation follow the same criteria described in the determinative method.

Record	*Acceptable Control*
DAD warmup	> 30 minutes
DAD zero	Prior to start of each injection (automatic with Chemstation software)

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Inject standard (4 μg/mL) to verify HPLC, DAD, and integration software operating parameters.

ii. Phase II (Optional—at discretion of supervisor): Analyze two sets of validation samples on separate days. Each set consists of a standard at 4 μg/mL, a reagent blank, 1 blank poultry liver, and 3 poultry liver blanks fortified at 4 ppm.

iii. Phase III: Analyze a standard at 4 ppm, a blank poultry liver, a blank liver fortified at 4 ppm, and 3 unknowns fortified at approximately 4 ppm.

b. Acceptability criteria.

The analyst must demonstrate the ability to meet confirmation criteria specified in sections F.3 and F.4 for all samples analyzed.

4. Intralaboratory Check Samples

a. System, minimum contents:

i. Frequency: One sample quarterly per analyst.

ii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer.

b. Acceptability criteria.

Ability to confirm samples using criteria specified in sections F.3 and F.4.

5. Sample Acceptability and Stability

a. Sample storage stability: Not determined.

b. Condition upon receipt: Cold.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

6. Sample Set	Refer to section F.2.
7. Sensitivity	a. Lowest detectable level (LDL): Not applicable. b. Lowest reliable confirmation (LRC): < 4 ppm.