NOVOBIOCIN AND VIRGINIAMYCIN

Contents

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory and Structures

The antibiotic compounds novobiocin (NOV) and virginiamycin (VIR) are used in the treatment of meat-producing animals. This method was developed to provide chemical identification of the antibiotics. Additionally, the method provides quantitative supporting data for the official biological method.

Residues are extracted from tissue by blending with methanol. The homogenate is centrifuged to remove solid material and an aliquot of the organic extract is removed and filtered for HPLC analysis of novobiocin. A second aliquot is transferred to a clean tube and processed for analysis of virginiamycin by extraction into dichloromethane. Both compounds are determined in a single gradient elution HPLC analysis on an LC-18-DB reversed phase LC column.

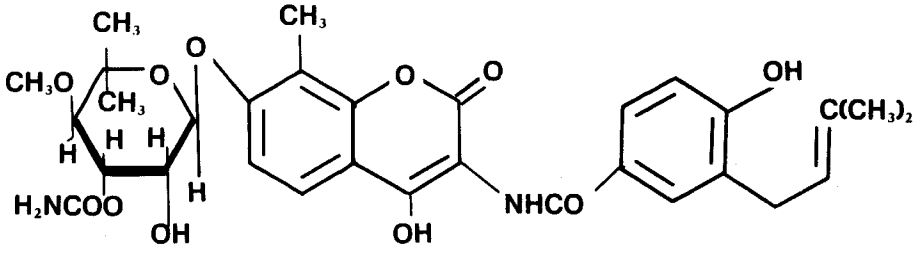
2. Applicability

This method is applicable to bovine, porcine, and avian species, and liver, muscle, or kidney tissue.

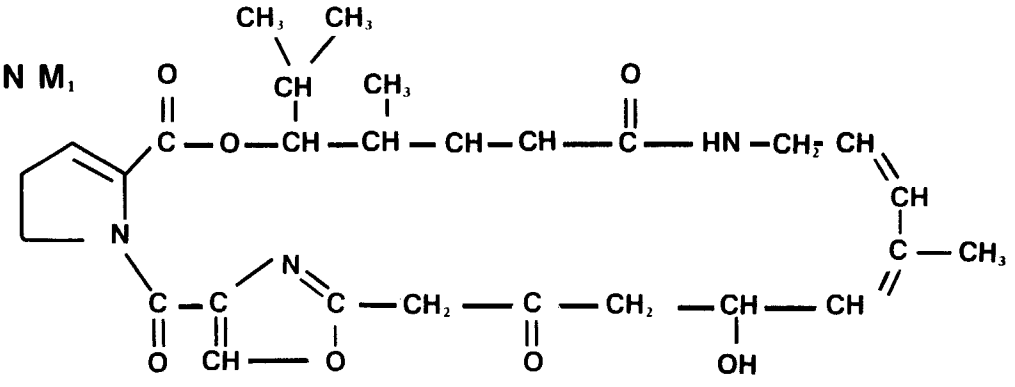
DETERMINATIVE METHOD

A. INTRODUCTION (Continued)

NOVOBIOCIN



VIRGINIAMYCIN M_1



DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Mechanical shaker: Eberbach reciprocating flat bed linear shaker.

b. N-Evap solvent removal system: Organomation Associates, Inc., Model 112, 24 place, or equivalent.

c. Centrifuge: Sorvall Model T600B, or equivalent.

d. Tissuemizer: Polytron Tissue Homogenizer, or equivalent.

e. 50 mL polypropylene centrifuge tubes: Falcon Blue Max, #2070, or equivalent.

f. Disposable syringe filters: Applied Science Series 6000 PTFE, 0.2 μm porosity, or equivalent.

2. Instrumentation

HPLC System: Hewlett Packard 1090 HPLC System equipped as follows:

i. Hewlett Packard Diode Array UV Detection System.

ii. Chem Station control hardware and software.

iii. Automatic injection system.

iv. Supelco LC-18 DB column, 15 cm × 4.6 mm i.d., 5 μm particle size (Supelco, Inc., catalog no. 5-8348888).

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

1. Reagent List

a. Methanol, Burdick and Jackson HPLC Grade, or equivalent.

b. Petroleum ether, Baker Resi-Analyzed Grade, or equivalent.

c. Methylene chloride (dichloromethane), Baker Resi-Analyzed Grade, or equivalent.

d. Acetonitrile, Burdick and Jackson HPLC Grade, or equivalent.

e. Phosphoric acid, Mallinckrodt Reagent Grade 85% (catalog no. 2761-1), or equivalent.

f. Ammonium phosphate, Mallinckrodt Reagent Grade (catalog no. 3476), or equivalent.

2. Solution List

a. 0.2M ammounium phosphate.

b. 0.01M phosphoric acid.

c. Acetonitrile:0.01M phosphoric acid—20:80.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

a. Novobiocin: Sigma Chemical Catalog No. N-1628, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO.

b. Virginiamycin-M1: Sigma Chemical Cat. No. V-2753, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO.

2. Preparation of Standards

a. Stock standard solutions (50 μg/mL).

i. Novobiocin—Accurately weigh 5.0 ± 0.1 mg noviobiocin reference standard material into a clean 100 mL volumetric flask. Dissolve and dilute to volume with HPLC-grade methanol.

ii. Virginiamycin—Accurately weigh 5.0 ± 0.1 mg virginiamycin reference standard material into a clean 100 mL volumetric flask. Dissolve and dilute to volume with HPLC-grade methanol.

b. Fortification standards.

i. Novobiocin—Stock standard solution will be used.

ii. Virginiamycin—Pipet 20 mL virginiamycin stock standard solution into a clean 100 mL volumetric flask and dilute to volume with HPLC-grade methanol to produce fortification standard solution. Concentration is 10 μg/mL.

c. HPLC reference standards.

i. Novobiocin.

(a) Pipet 20 mL novobiocin stock standard solution into a clean 100 mL volumetric flask and dilute to volume with HPLC-grade methanol. Concentration is 10 μg/mL.

(b) Pipet 1.66 mL novobiocin solution (a) above into a clean 100 mL volumetric flask and dilute to volume with methanol. Concentration is 0.166 μg/mL, equivalent to 0.5 ppm tissue concentration.

(c) Pipet 3.33 mL novobiocin solution (a) above into a clean 100 mL volumetric flask and dilute to volume with methanol. Concentration is 0.333 μg/mL, equivalent to 1.0 ppm tissue concentration.

(d) Pipet 6.66 mL novobiocin solution (a) above into a clean 100 mL volumetric flask and dilute to volume with methanol. Concentration is 0.666 μg/mL, equivalent to 2.0 ppm tissue concentration.

ii. Virginiamycin.

(a) Pipet 2 mL virginiamycin fortification standard solution into a clean 50 mL volumetric flask and dilute to volume with HPLC-grade methanol. Concentration is 0.4 μg/mL, equivalent to 0.1 ppm tissue concentration.

DETERMINATIVE METHOD

D. STANDARDS (Continued)

	ii. Pipet 4 mL virginiamycin fortification standard solution into a clean 50 mL volumetric flask and dilute to volume with HPLC-grade methanol. Concentration is 0.8 μg/mL, equivalent to 0.2 ppm tissue concentration.
	iii. Pipet 8 mL virginiamycin fortification standard solution into a clean 50 mL volumetric flask and dilute to volume with HPLC-grade methanol. Concentration is 1.6 μg/mL, equivalent to 0.4 ppm tissue concentration.
3. Storage Conditions	Stock solutions should be stored tightly stoppered at 4° C.
4. Shelf Life Stability	Stock solutions are stable up to 6 months if stored as described above.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Procedure

a. Weigh 5.0 ± 0.1 g homogenized tissue into a clean 50 mL polypropylene centrifuge tube.

NOTE: Recovery samples for novobiocin are fortified using the novobiocin stock standard solution as follows: 1.0 ppm tissue level, add 100 µL novobiocin stock solution.

Recovery samples for virginiamycin are fortified using the virginiamycin fortification standard solution as follows: 0.2 ppm tissue level, add 100 µL virginiamycin fortification solution.

b. Add 15 mL HPLC-grade methanol and blend using a Polytron Tissuemizer or similar blending device at medium speed for 1 minute.

c. Centrifuge samples for 5 minutes at 2000 rpm (1000 × G).

d. Remove 1 mL of the clear methanol layer and pass through a 0.2 µm syringe filter, collecting filtrate in an HPLC auto sampler vial. Cap sample and reserve for analysis of novobiocin by HPLC.

e. Remove 6 mL of the clear methanol layer and place in a clean 50 mL polypropylene centrifuge tube. Add 5 mL 0.2M $NH_4H_2PO_4$ and 5 mL petroleum ether. Cap samples and shake vigorously for 30 seconds using a flat-bed reciprocating linear shaker.

f. Centrifuge for 5 minutes at 2000 rpm (1000 × G), aspirate, and discard the petroleum ether layer.

g. Add 5 mL petroleum ether and 25 mL dichloromethane, cap, and shake vigorously for 2 minutes, using mechanical shaker.

h. Aspirate and discard the upper organic layer.

i. Add 0.6 mL distilled water and 0.6 mL acetonitrile. Reduce volume to approximately 0.3 mL, using an N-Evap solvent evaporation device with water bath set at 50° C and a gentle stream of dry nitrogen.

j. Add 12 mL HPLC-grade acetonitrile, carefully rinsing the sides of the tube. Evaporate to dryness using the N-Evap.

k. Redissolve residue in 500 µL of the HPLC mobile phase (20:80 acetonitrile:0.01M phosphoric acid) and transfer to an HPLC autosampler vial. Cap samples and reserve for analysis by HPLC.

2. Screening Test

Since it is anticipated that the vast majority (approaching 100%) of all samples analyzed by this method will be negative, it is efficient to screen all sample extracts prior to quantitative analysis. To perform the screening test, the following sequence should be followed:

a. Inject 25 µL of the novobiocin/virginiamycin mixed HPLC reference standard, followed by the novobiocin sample extracts and then the virginiamycin sample extracts.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

b. Evaluate the resulting chromatograms for tentative positive findings. A sample is identified as tentatively positive if both of the following conditions are met:

i. There is a peak within ± 0.1 min of the retention time of either the novobiocin or virginiamycin reference peak from the standard injection.

ii. The peak in question is ≥ 20% of the peak height of the corresponding reference standard.

3. Preparation of Standard Curve

If both of the conditions in E.2.b are met, the sample is identified as tentatively positive. If such a case exists, the following quantitative determination should be performed in order to produce a standard curve:

a. Inject 25 μL of the appropriate low level HPLC standard (0.166 μg/mL for novobiocin and 0.4 μg/mL for virginiamycin).

b. Inject 25 μL of the appropriate middle level HPLC standard (0.333 μg/mL for novobiocin and 0.8 μg/mL for virginiamycin).

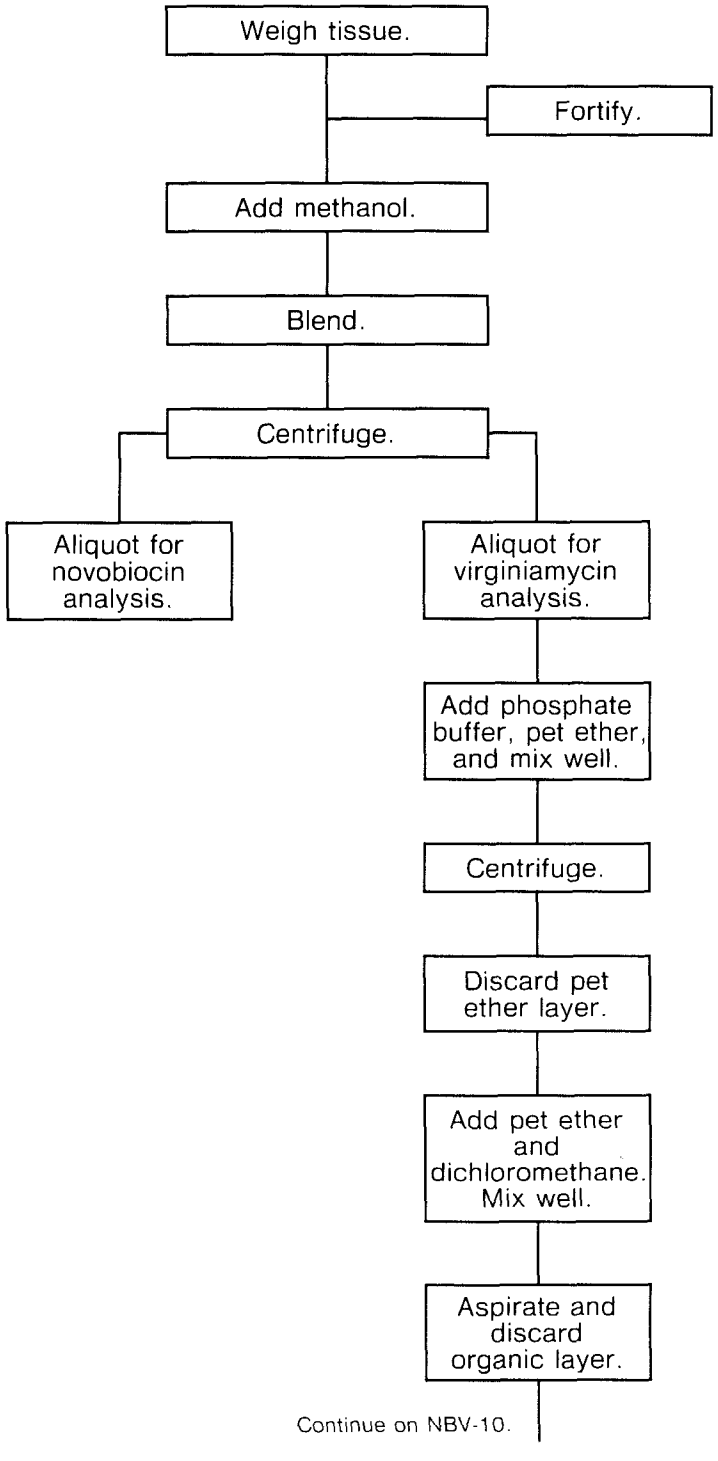
c. Inject 25 μL of the appropriate high level HPLC standard (0.666 μg/mL for novobiocin and 1.6 μg/mL for virginiamycin).

d. Inject 25 mL of each tentatively positive sample.

DETERMINATIVE METHOD

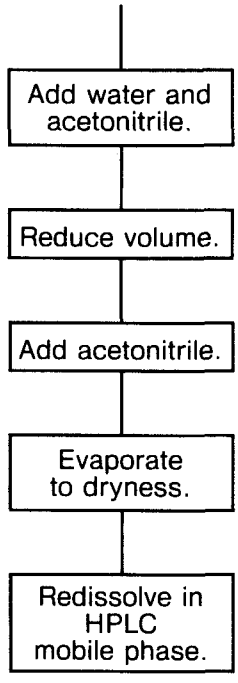
E. EXTRACTION PROCEDURE (Continued)

4. Flow Chart Summary



DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)



DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

Instrumental Settings and Conditions

HPLC conditions.

a. Column: Supelco LC-18 DB Column, 15 cm length, 4.6 mm i.d., 5 μm particle size.

b. Flow rate: 1.0 mL/min

c. Elution system: A gradient analysis is used in order to analyze both novobiocin and virginiamycin in a single injection. The gradient profile is as follows:

Time	*Solvent A(%)*	*Solvent B(%)*
0.2	94.0	6.0
30.0	15.0	85.0
35.0	15.0	85.0
40.0	94.0	6.0
45.0	94.0	6.0

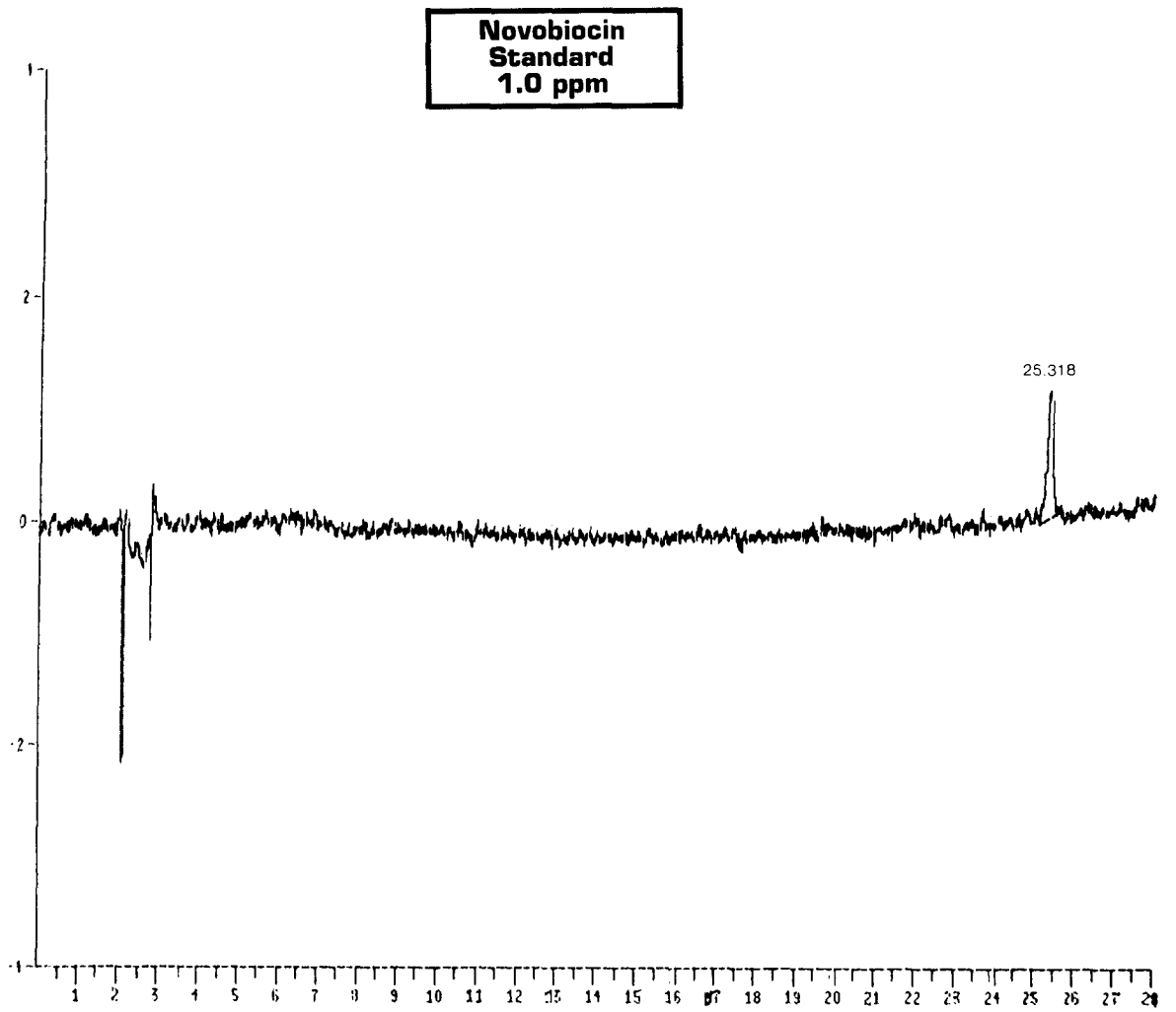
Where Solvent A = 0.01M phosphoric acid
Solvent B = acetonitrile

d. Diode array detector settings:

	Novobiocin	*Virginiamycin*
Wavelength	340 nm	230 nm
Bandwith	10 nm	10 nm
Reference wavelength	550 nm	550 nm
Reference bandwith	10 nm	10 nm
Sampling interval	640 ms	640 ms
Spectrum range	210-400 nm	210-400 nm

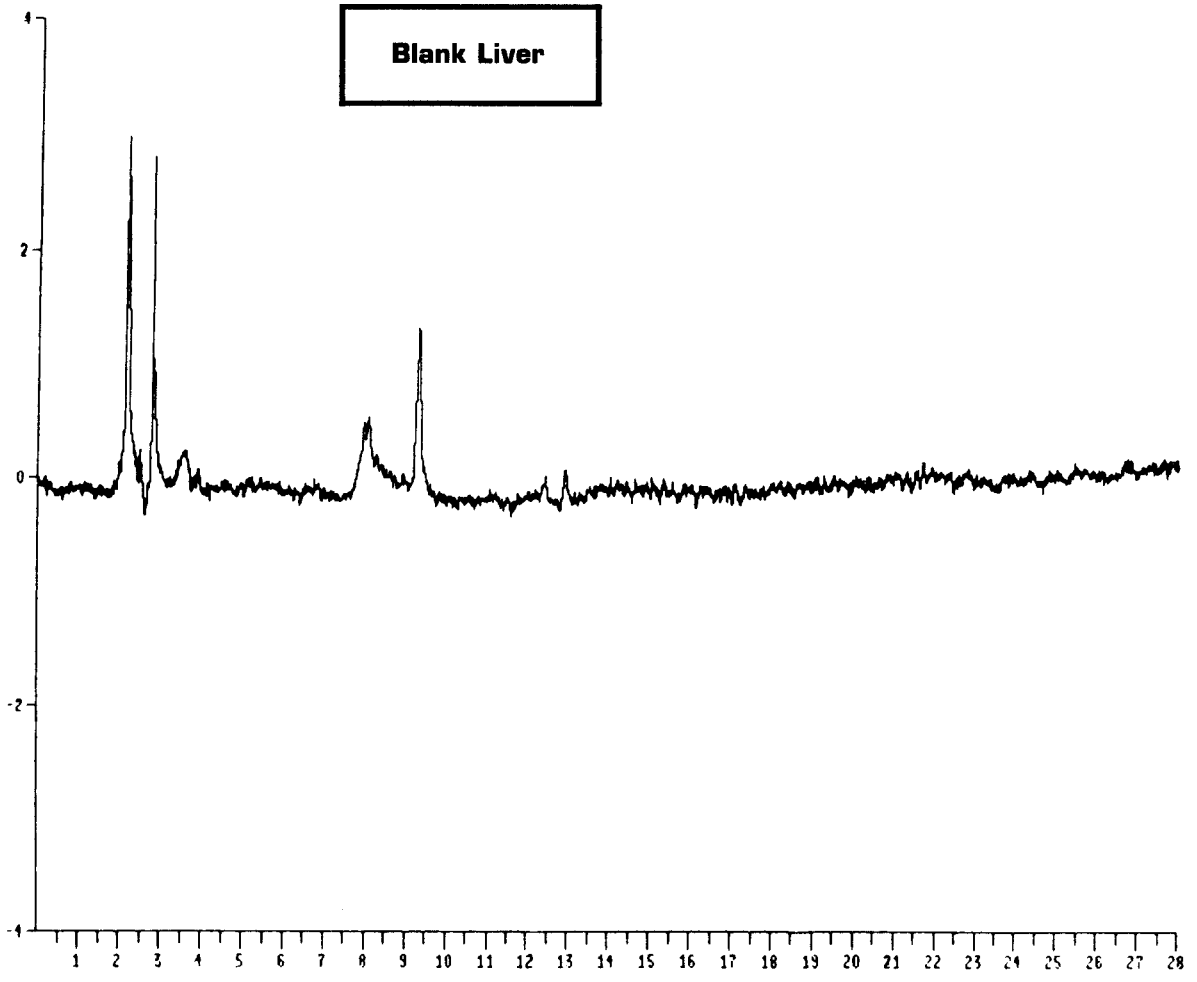
DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



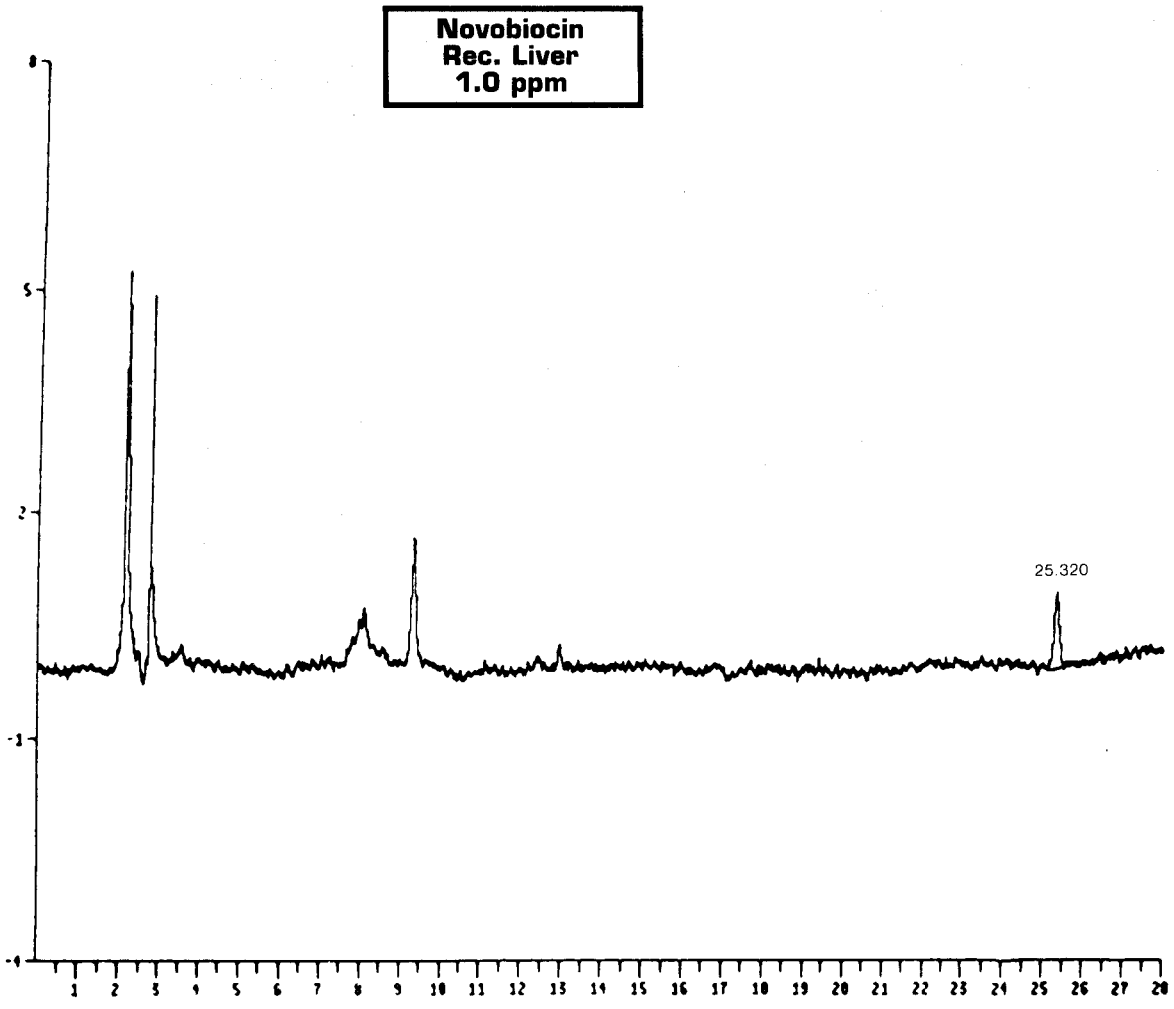
DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



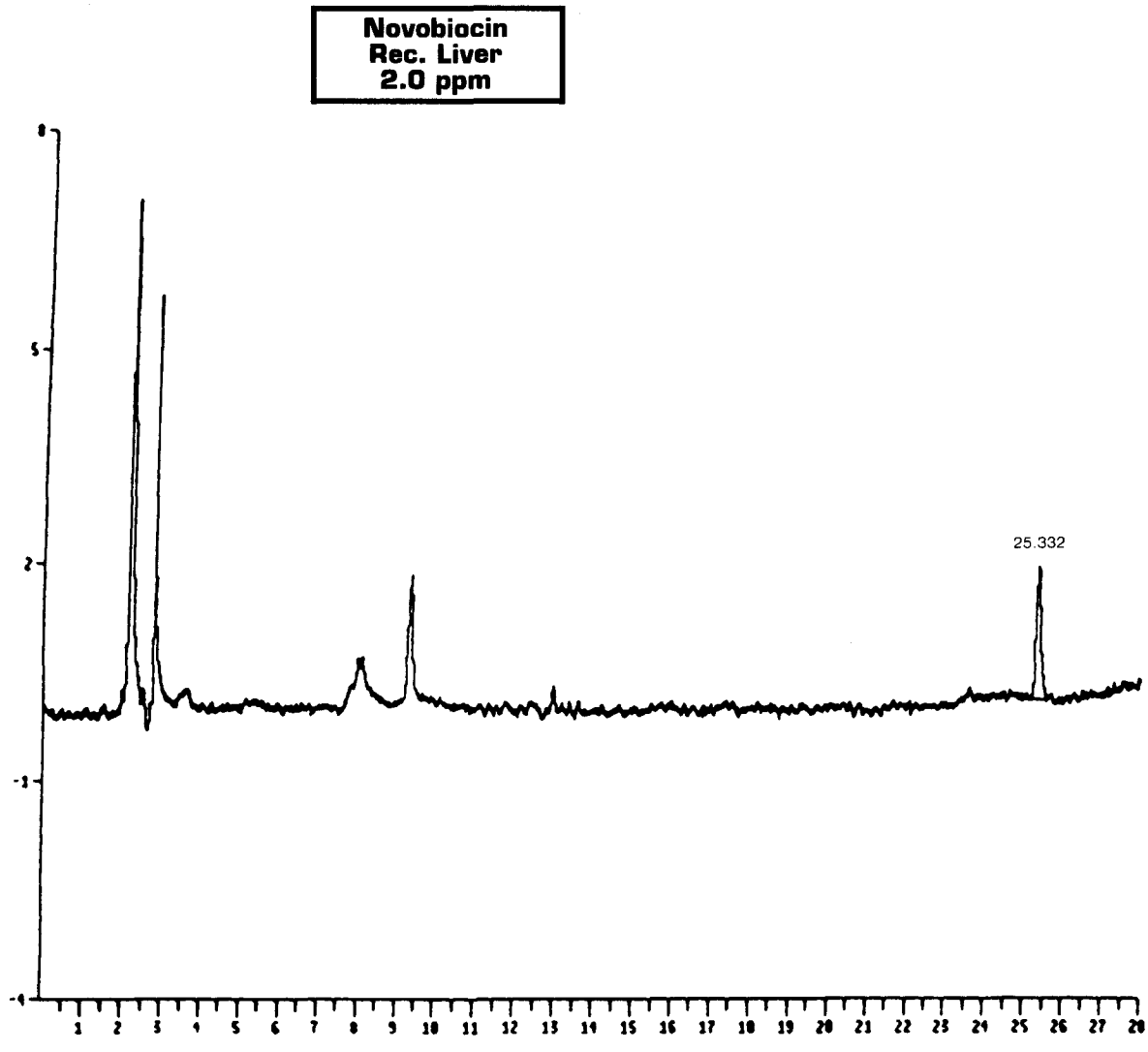
DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



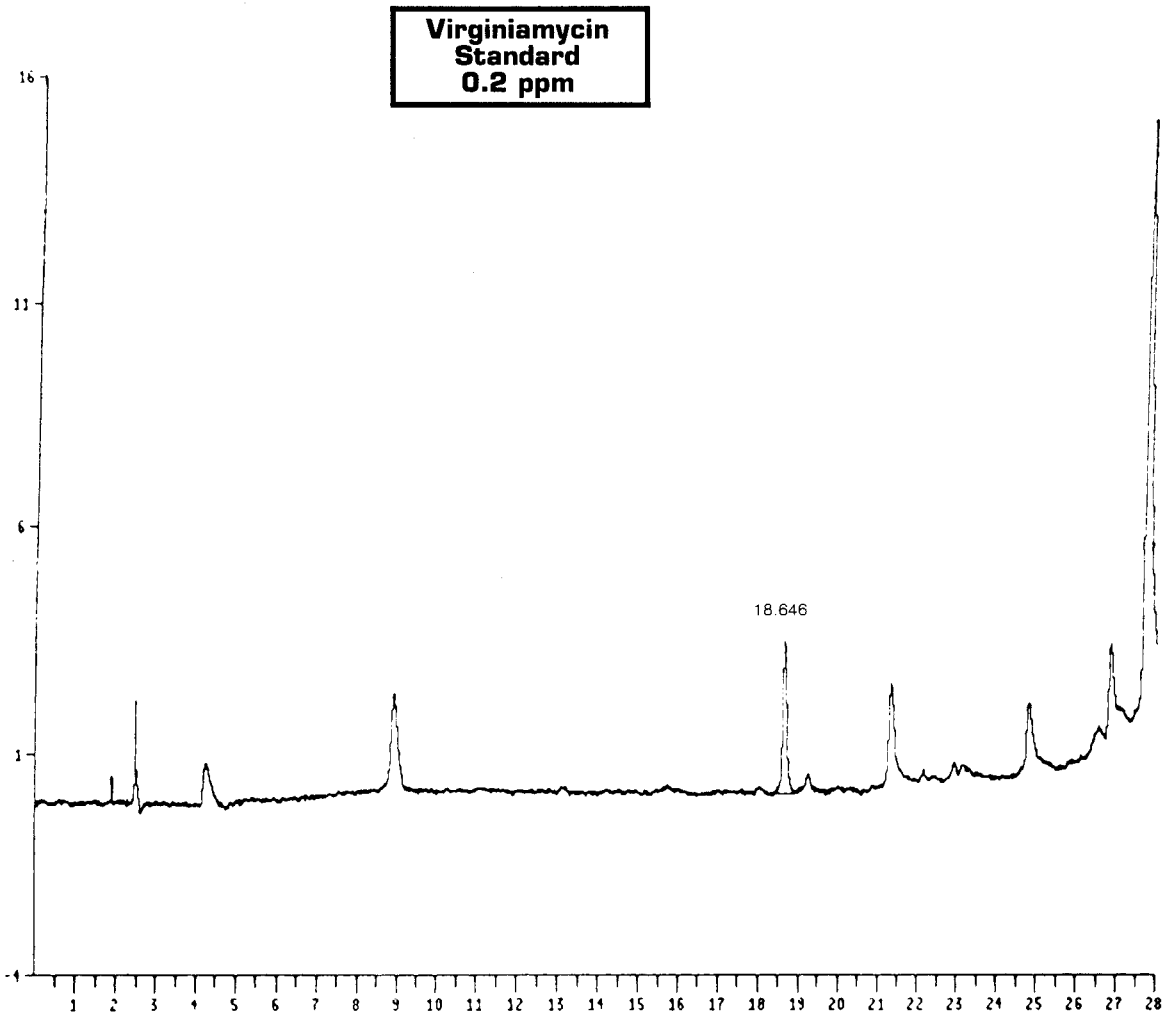
DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



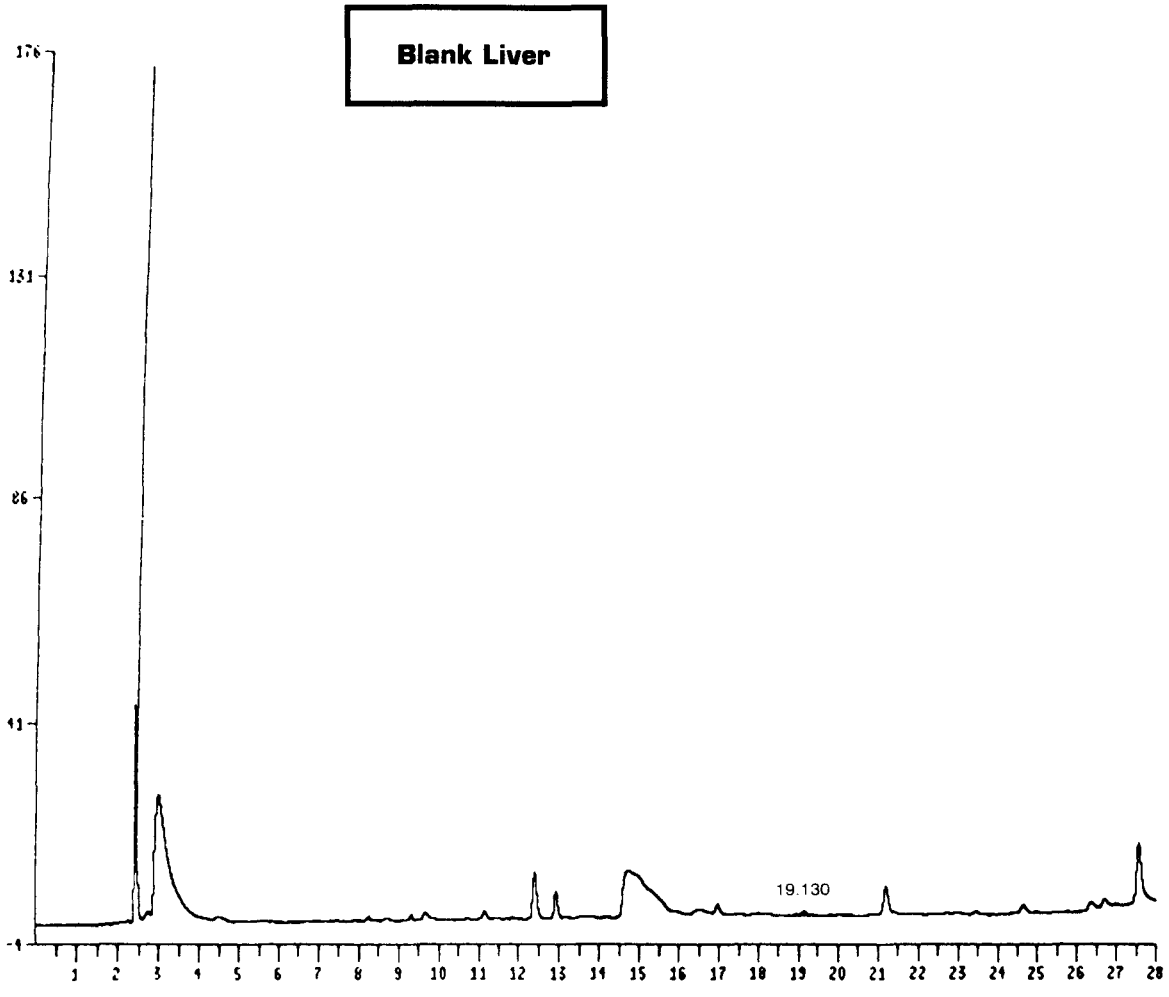
DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



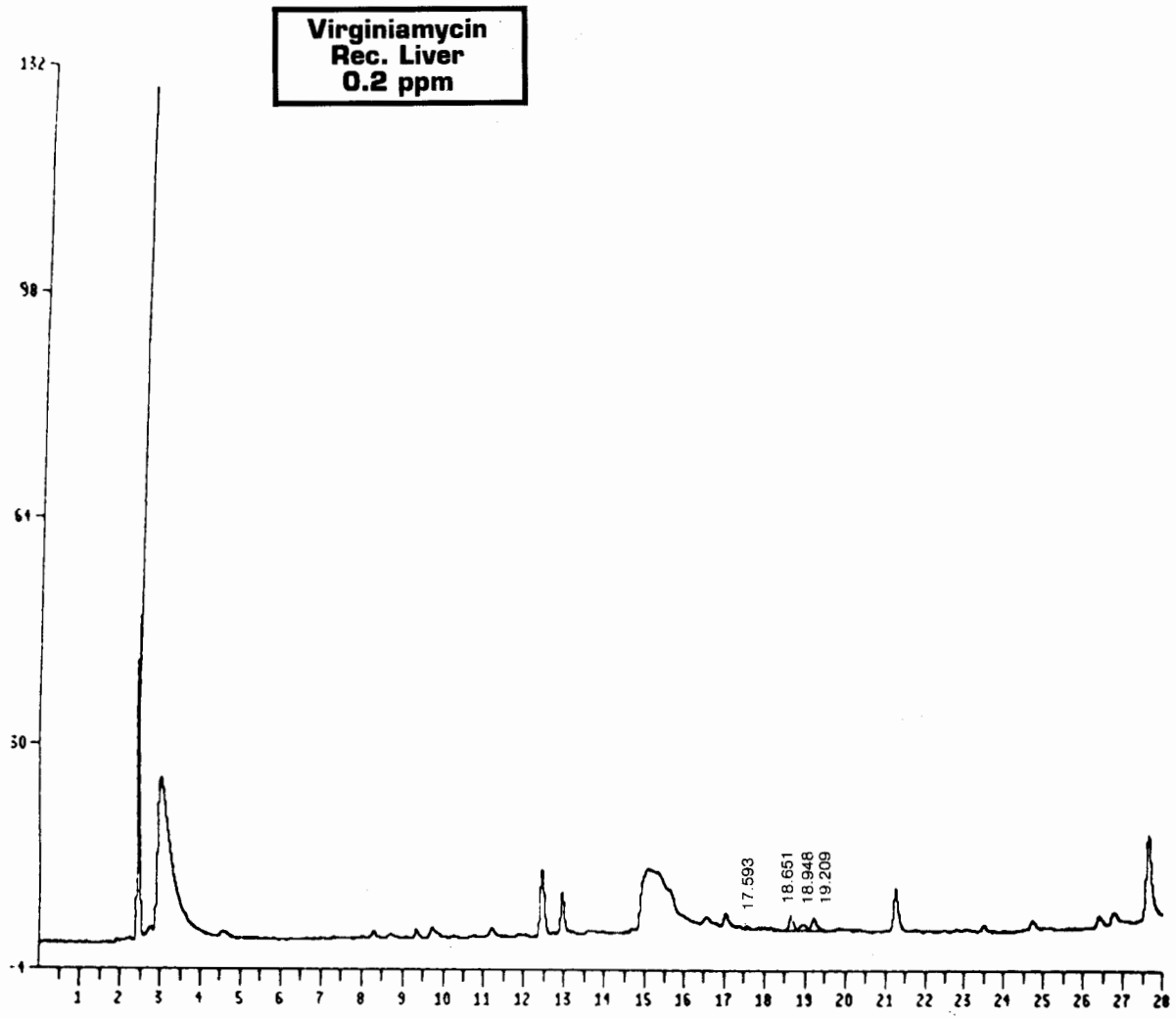
DETERMINATIVE METHOD

F. ANALYTICAL QUANTIATION (Continued)



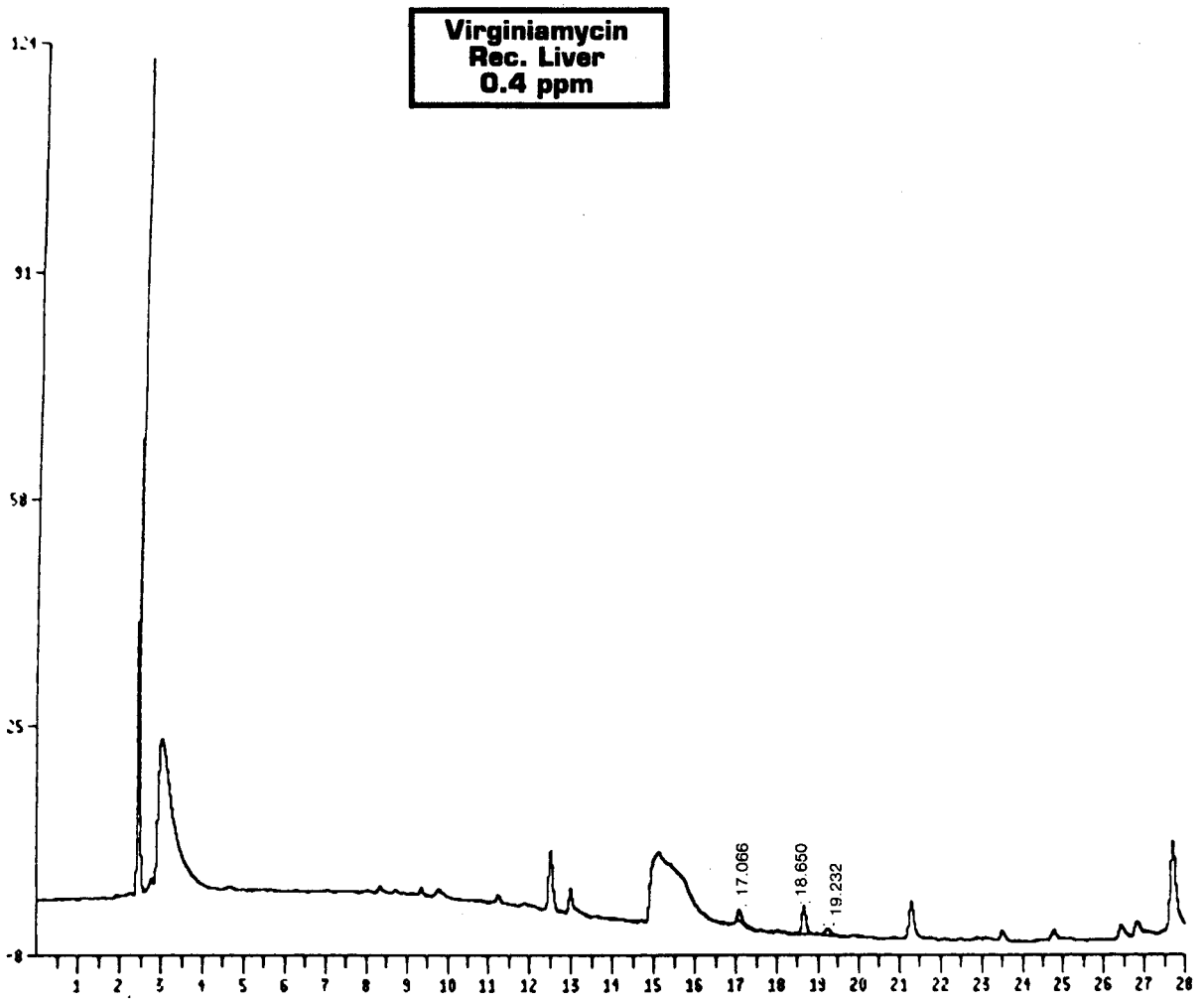
DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

By an acceptable means, measure the peak heights of the 0, 0.5, 1.0, and 2.0 ppm novobiocin standards. By an acceptable means, measure the peak heights of the 0, 0.1, 0.2, and 0.4 ppm virginiamycin standards. Using peak height and associated ppm values, construct a standard calibration curve by least squares calibration, as indicated in the Chemistry Quality Assurance Handbook Volume I, page 1.5.67. Using the standard curve, determine the concentration of novobiocin or virginiamycin in the sample.

Determine percent recovery for the fortification standard. Use the 10-day running average to correct for recovery. The 10-day running average should be determined for each species/matrix combination. After it can be shown that there is no species/matrix bias on recoveries, the 10-day running averages do not have to be maintained for each species/matrix combination.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Analysis of Novobiocin and Virginiamycin in Animal Tissues		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.		
3. Procedure Steps		*Hazards*	*Recommended Safe Procedures*
	C. Reagents		
	Acetonitrile Petroleum ether Methanol Methylene chloride	These solvents are all flammable and may produce toxic effects to the skin, eyes, and respiratory system. Vapors are hazardous.	Work in efficient fume hood, away from electric heaters. Use plastic gloves.
	Phosporic acid	Corrosive.	Wear eye protection, lab coat, and protective gloves.
4. Disposal Procedures	Organic solvents	See above	Store in organic solvent disposal container until disposed of by a contractor or in-house specialist.
	Acidic solutions	See above	Dilute and flush into an acid disposal sink located in a well-ventilated area.
	Methylene chloride	See above	Store with waste chlorinated solvents until disposed of by a contractor or in-house specialist.

DETERMINATIVE METHOD

I. WORKSHEET

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

NOVOBIOCIN AND VIRGINIAMYCIN

SAMPLE NUMBER:

TISSUE:

ANALYST:

DATE:

Sample	*Retention Time*	*Peak Height/Area* *Novobiocin 340nm*	*Virginiamycin 230 nm*
Blank			
Standard			

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound	*Analytical Range (ppm)*	*Acceptable Recovery (%)*	*Repeatability %CV*	*Reproducibility %CV*
Novobiocin	0-10.0	60-100	≤ 10.0	≤ 15.0
Virginiamycin	0- 4.0	60-100	≤ 10.0	≤ 15.0

2. Critical Control Points and Specifications

Record	*Acceptable Control*
Sample weight	5.0 g ± 0.1 g
Volume of extraction solvent	15.0 mL ± 0.2 mL

3. Readiness To Perform

a. Familiarization

i. Novobiocin.

(a) Phase I: Duplicate sets of standard curves on each of three days at 0, 0.5, 1.0, and 2.0 ppm.

(b) Phase II: Self-fortified samples using beef liver tissue spiked at 0, 0.5, 1.0, and 2.0 ppm (duplicates) on three successive days.

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

(c) Phase III: Check samples for analyst accreditation.

(1) Twelve samples submitted by laboratory LSO.

(2) Report findings through LSO to Chemistry Division, QSB.

Letter from Chemistry Division is required to commence official analysis.

ii. Virginiamycin.

(a) Phase I: Duplicate sets of standard curves on each of three days at 0, 0.1, 0.2, and 0.4 ppm.

(b) Phase II: Self-fortified samples using beef liver tissue spiked at 0, 0.1, 0.2, and 0.4 (duplicates) on three successive days.

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

(c) Phase III: Check samples for analyst accreditation.

(1) Twelve samples submitted by laboratory LSO.

(2) Report findings through LSO to Chemistry Division, QSB.

Letter from Chemistry Division is required to commence official analysis.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

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. Blind samples or random duplicates chosen by the supervisor or LSO.

ii. Records to be maintained by analyst and reviewed by supervisor and LSO.

(a) All replicate findings.

(b) CUSUM charts.

(c) All recovery values.

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

b. Acceptability criteria.

If unacceptable values are obtained, then:

(a) Stop all official analyses for that analyst.

(b) Investigate and identify probable cause.

(c) Take corrective action.

(d) Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrices: Liver, muscle, or kidney.

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

c. Condition upon receipt: Chilled or frozen.

d. Sample storage:

i. Time: 3 months.

ii. Condition: Store frozen at $-4°$ C or lower until analyzed.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

6. Sample Set

a. Blank.

b. Fortification standard: 1.0 ppm novobiocin; 0.2 ppm virginiamycin.

c. Samples.

7. Sensitivity

a. Lowest detectable level (LDL).

i. Novobiocin: 0.5 ppm.

ii. Virginiamycin: 0.1 ppm.

b. Lowest reliable quantitation (LRQ).

i. Novobiocin: 1.0 ppm.

ii. Virginiamycin: 0.2 ppm.

c. Minimum proficiency level (MPL).

i. Novobiocin: 1.0 ppm.

ii. Virginiamycin: 0.2 ppm.