

MONENSIN IN TISSUES

Contents

A. Introduction	1
B. Equipment	2
C. Reagents and Solutions	3
D. Standards	5
E. Extraction Procedure	6
F. Analytical Quantitation	11
G. [Reserved]	
H. [Reserved]	
I. [Reserved]	
J. [Reserved]	

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Monensin is extracted from experimental samples with methanol. Tissue extracts are processed by carbon tetrachloride or by silica gel column chromatography. Aliquots of the semipurified extracts in methanol are subjected to thin-layer chromatography on silica gel plates, and the antibiotic is detected on the TLC plates by bioautographic techniques using *Bacillus subtilis* as the assay organism. Monensin levels are estimated by comparing zones of inhibition from experimental samples to those from standard recoveries. Samples are authenticated by Polaroid photography.

2. Applicability

This method is applicable to chicken fat only.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. Tissue blender: Hamilton Beach Model 8, or equivalent, equipped with blender heads to fit half-pint Ball Mason jars.
 - b. Centrifuge: Servall Model NSE, or equivalent.
 - c. Rotary vacuum evaporator: Rinco evaporator, or equivalent.
 - d. Thin-layer chromatographic developing chambers: Brinkmann Instruments, Inc., Westbury, NY, or equivalent.
 - e. Photographic equipment: Polaroid Model MP 3, or equivalent.
 - f. Incubator maintained at 37° C.
 - g. Colorimeter: Spectronic 20, or equivalent.
 - h. Water bath.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. Hexane A.R.
 - b. Methanol A.R.
 - c. Chloroform A.R.
 - d. Benzene A.R.
 - e. Carbon tetrachloride A.R.
 - f. Ethylene glycol monomethyl ether A.R.
 - g. Chloroform A.R., containing 5% by volume of methanol A.R.
 - h. Silica gel G for thin-layer chromatography, Brinkmann Instruments, Inc., Westbury, NY, or equivalent.
 - i. Silica gel 0.2 to 0.5 mm for column chromatography, Brinkmann Instruments, Inc., or equivalent.
 - j. Glass wool.
 - k. Sodium sulfate anhydrous A.R. (granular).
 - l. Agar medium: Dissolve 0.69 g $K_2HPO_4 \cdot 3H_2O$, 0.45 g KH_2PO_4 , 2.5 g yeast extract (Difco), 10 g glucose (cerelose), and 6.0 agar in enough deionized water to give 1 L total volume. Autoclave the solution for 20 min at 121° C.
 - i. Ion agar, 2S (Colab Laboratories), or equivalent.
 - ii. Noble agar (Difco), or equivalent.
 - iii. Agar granulated, No. 11849 (BBL), or equivalent. Use at 1% concentration.
 - m. The following two commercially prepared plates or their equivalent can be used.
 - i. Q47G silica gel TLC plates, Quantum Industries, 341 Kaplan Drive, Fairfield, NJ.
 - ii. Woelm silica gel G TLC plates, Analtech, Inc., 75 Blue Hen Drive, Newark, DE.
 - iii. Laboratory-prepared thin-layer chromatography plates can be used. Prepare a slurry of 26 g silica gel G in 55 mL of deionized water and apply to standard 20 × 20 cm glass plates at a thickness of 250 microns. Air-dry the plates for 10-15 min. Activate the plates in a drying oven by heating at 50° C for 15 min, 90° C for 15 min, and finally 110° C for 30 min. Following activation, allow the plates to cool slowly to room temperature.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS (Continued)

- n. *Bacillus subtilis* stock solution.
- i. Wash the growth of *Bacillus subtilis*, American Type Culture Collection, No. 6633, from one penassay seed agar slant (culture medium No. 1, Grove and Randall, Assay Methods of Antibiotics) with 3-5 mL sterile distilled water onto seeded agar with 0.03% manganous sulfate added.
 - ii. Incubate the culture for one week at 37° C.
 - iii. Following incubation, wash the growth from the agar surface of the Roux bottle with approximately 50 mL of sterile distilled water.
 - iv. Transfer the wash to a sterile 250 mL centrifuge bottle and hold the organisms at 65° C in a water bath for 30 min.
 - v. Centrifuge the suspension and discard the supernatant liquid.
 - vi. Repeat the resuspension and washing of the organisms three times.
 - vii. After the final wash, heat-shock the cells again by immersion in the 65° C water bath for 30 min and suspend them in 30 mL sterile deionized water.

NOTE: This stock suspension is stored at 4°-5° C for use.

- o. *Bacillus subtilis* inoculum: Prepare the inoculum fresh weekly by diluting the stock suspension with sterile distilled water to obtain a 20% light transmittance at 530 nm using a Spectronic-20 colorimeter.
 - p. A 2 mg/mL solution of INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride) in 10% methanol:distilled water.
-

DETERMINATIVE METHOD

D. STANDARDS

1. Preparation of Standards

Standard solutions.

- a. Weigh a quantity of standard sodium salt of monensin to contain 50 mg of monensin activity.
- b. Transfer the standard quantitatively to a 50 mL volumetric flask and dissolve the salt in methanol. Dilute to the mark with methanol and mix thoroughly. This standard solution contains 1000 $\mu\text{g/mL}$ of monensin activity.
- c. Pipette 1 μL of 1000 $\mu\text{g/mL}$ standard into a 200 mL volumetric flask with a volumetric pipette. Dilute to the mark with methanol and mix thoroughly. This standard contains 5 $\mu\text{g/mL}$ of monensin activity.

2. Storage Conditions

Stock solutions should be stored tightly stoppered at 4° C.

3. Shelf Life Stability

Standard solutions are stable at least 10 days when stored under refrigeration as described in section 3.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Lean, Liver, and Kidney Tissue Extraction and Purification

-
- a. Weigh a representative ground or minced tissue sample (approximately 30 g) into a half-pint extraction jar.
 - b. Add 2 mL methanol per gram of tissue.
 - c. Blend sample until uniform.
 - d. Transfer sample to centrifuge tubes and centrifuge at -10° for 10-15 min (maximum rating of Servall Model NSE, 7500 rpm). (A refrigerated centrifuge is recommended to aid in removal of lipids.)
 - e. Transfer a measured volume of supernate (ca 1.5 mL/g tissue) to a 250 mL separatory funnel.

NOTE: In a series of samples, calculation may be expedited by using a uniform aliquot size based on the maximum amount of supernate obtained from the smallest sample.

- f. Extract the supernate with three 30 mL portions of carbon tetrachloride. Combine the CCl_4 fractions in a 300 mL evaporating flask and evaporate to dryness by rotary vacuum evaporation, using a water bath at 50° - 60° C.

NOTE: Approximately 2 min is required to complete the carbon tetrachloride extraction on each sample. Shake the MeOH extract and CCl_4 thoroughly and allow complete separation to occur before removing the CCl_4 fractions.

- g. Transfer the sample with 3 mL of hexane to a 15 mL glass-stoppered sample tube. Rinse the flask with an additional 2 mL of hexane and transfer the rinse to the sample tube.

NOTE: Transfer can be made by pipette or by pouring carefully from the evaporating flask.

- h. Evaporate the hexane using an N-Evap at 45° C under nitrogen.
- i. Dissolve the sample in methanol using 0.05 mL methanol per 10 mL of supernate extracted.

2. Fat Tissue Extraction and Purification

-
- a. Weigh a ground fat sample of approximately 20 g into a half-pint extraction jar.
 - b. Add 5 mL methanol per gram of fat and blend the sample until uniform.
 - c. Centrifuge sample for 10-15 min.
 - d. Transfer the supernate to a 300 mL evaporating flask and evaporate to dryness *in vacuo* (rotary vacuum evaporator) at 50 - 60° C.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

-
- e. Prepare silica gel column for chromatography.
 - i. Place ca 10 mL hexane into a 14 × 250 mm (250 mL reservoir) glass chromatographic column. Insert a glass wool pledget and tamp with a glass stirring rod to eliminate air bubbles.
 - ii. Add 2.4 g (5 mL measured with a 10 mL graduated cylinder) of 0.2-0.5 mm silica gel to the column through a powder funnel and follow with ca 5 mL hexane.
 - iii. Stir the silica gel with a glass rod to eliminate air bubbles; then let the column stand until the silica gel settles. Drain a small volume of hexane through the column to facilitate packing.
 - iv. Add ca 2 cm of anhydrous sodium sulfate to the column, layering it carefully to avoid disturbance of the silica gel surface.
 - v. Drain the hexane to the top of the sodium sulfate.
 - vi. Prepare a separate column for each sample.
 - f. Dissolve the sample from step d in 10 mL hexane and charge the silica gel column with this solution at a flow rate of ca 3 mL/min. Rinse the evaporating flask with an additional 10 mL hexane and transfer the rinse to the column. Discard the hexane effluent.
 - g. Develop the silica gel column with approximately 100 mL chloroform at a flow rate of ca 3 mL/min. Discard the chloroform effluent.
 - h. Place an evaporating flask in position to receive the column effluent.
 - i. Elute the monensin from the column with 30 mL of chloroform containing 5% methanol.
 - j. Evaporate the eluate to dryness and transfer the sample to sample bottles as described in steps 1.g and 1.h.
 - k. Dissolve the sample in 0.1 mL methanol per 10 g of fat in the original sample.
-

**3. Liver Tissue
Extraction and
Purification—
Alternate
Determination**

This procedure is recommended if fat from liver samples interferes with the thin-layer chromatographic separation of monensin in steps 1.a-1.i.

- a. Weigh a representative ground or minced tissue sample (approximately 30 g) into a half-pint extraction jar.
- b. Add 2 mL methanol per gram of tissue.
- c. Blend sample until uniform.
- d. Transfer sample to centrifuge tubes and centrifuge at room temperature for 10-15 min (maximum rating of Servall Model NSE, 7500 rpm).

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

-
- e. Transfer a measured volume of supernate (ca 1.5 mL/g tissue) to a 250 mL separatory funnel.

NOTE: In a series of samples, calculations may be expedited by using a uniform aliquot size based on the maximum amount of supernate obtained from the smallest sample.

- f. Extract the supernate with three 30 mL portions of carbon tetrachloride. Combine the CCl₄ fractions in a 300 mL evaporation flask and evaporate to dryness by rotary vacuum evaporator using a water bath at 50°-60° C.

NOTE: Approximately 2 min is required to complete the carbon tetrachloride extraction on each sample. Shake the MeOH extract and CCl₄ thoroughly and allow complete separation to occur before removing the CCl₄ fractions.

- g. Prepare silica gel column for chromatography as described for fat samples in step 2.e.
- h. Dissolve the sample from step f in 10 mL hexane and charge the silica gel column with this solution at a flow rate of ca 3 mL/min. Rinse the evaporating flask with an additional 10 mL hexane and transfer the rinse to the column. Discard the hexane effluent.
- i. Develop the silica gel column with approximately 100 mL chloroform at a flow rate of ca 3 mL/min. Discard the chloroform effluent.
- j. Place an evaporating flask in position to receive the column effluent.
- k. Elute the monensin from the column with 30 mL of chloroform containing 5% methanol.
- l. Evaporate the eluate to dryness and transfer the sample to sample bottles as described in steps 1.g and 1.h.
- m. Dissolve the sample in methanol using 0.05 mL methanol per 10 mL of supernate extracted.

4. Preparation of Standard Recoveries

Prepare negative control samples and standard recoveries for assay with each set of experimental samples.

NOTE: Prior to assaying samples, determine the linear range in recovery responses by testing several recovery samples. These samples should include those concentrations specified below in step b.

- a. Weigh appropriate control tissue into half-pint blender jars.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

-
- b. Add standard monensin (5 $\mu\text{g}/\text{mL}$ in methanol) by pipette to give the desired concentration in the recovery samples.

NOTE: One negative control (no monensin added), one recovery sample containing 0.04 $\mu\text{g}/\text{g}$ wet tissue (0.04 ppm), one recovery sample containing 0.05 $\mu\text{g}/\text{g}$ (0.05 ppm), and one recovery sample containing 0.06 $\mu\text{g}/\text{g}$ (0.06 ppm) are generally processed with each set of experimental samples. However, additional recovery levels may be included if desired.

Control and recovery samples are extracted and chromatographed exactly as described for the corresponding tissue.

5. Thin-Layer
Chromatography

Chromatograph the extracts from sections 1-4 above on thin-layer plates as follows:

- a. Apply 20 μL of methanol extract of negative control sample, standard recovery samples, and experimental samples to a silica gel thin-layer plate. A forced-air hair dryer may be used to hasten evaporation of solvent during sample application.

NOTE: Eight or nine samples may be assayed on one thin-layer plate.

- b. Apply negative control and recovery samples to each thin-layer plate in order to ensure adequate standardization of the procedure.
- c. Label the TLC plate and draw a line in the silica gel with a pencil or a scribe 3 cm from the top of the plate to mark the solvent front.
- d. Line a TLC developing chamber with a sheet of filter paper to improve saturation of the atmosphere in the chamber.
- e. Prepare the developing solvent by mixing 80 parts carbon tetrachloride, 10 parts benzene, and 6 parts ethylene glycol monomethyl ether. Prepare solvent system daily and allow to equilibrate prior to plate development.
- f. Pour the solvent down the sides of the developing chamber in order to saturate the filter paper while charging the chamber.
- g. Develop the thin-layer plate in the chamber, allowing the solvent front to move up to the line previously scored on the plate.

NOTE: Development time is about 75 min.

- h. Remove the plate from the chamber and allow the plate to air-dry on the laboratory bench. All traces of solvent should be removed from the plate.
-

6. Preparation of
Bioautographs

Bioautographs are prepared according to the method of Kline and Golab, *J. Chromatog.*, 18, 409 (1965).

- a. Melt agar medium (refer to section C, Reagent and Solution List, item I) in a steam bath.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

-
- b. Pour approximately 50 mL of melted agar into a 250 mL Erlenmeyer flask and fix the TLC plate by spraying the surface with the agar, using an atomizer-type sprayer attached to the laboratory compressed air supply.

NOTE: An artist's paint sprayer (Grumbacher atomizer #836, or equivalent) is satisfactory. Only a thin layer of agar should be sprayed on the TLC plate.

- c. Place the TLC plate in a plexiglass holder (or other suitable device that will prevent contamination of the plate) and allow the plate to come to room temperature.

NOTE: This step is necessary to prevent too rapid solidification of the seeded agar overlay.

- d. Cool 50 mL of melted agar medium to $54^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in a 125 mL Erlenmeyer flask.

NOTE: Cool slowly to prevent solidification of the agar before it can be inoculated and poured.

- e. Inoculate the agar with 0.05 to 0.1 mL of *B. subtilis* inoculum (refer to section C, Reagent and Solution List, item o). Mix quickly by swirling.

NOTE: The inoculum may be adjusted to give adequate test sensitivity.

- f. Pour this seeded agar over the surface of the TLC plate.

NOTE: Pouring of the seed agar must be done rapidly and carefully to ensure an even agar overlay before the agar solidifies. The plate may be slightly warmed to 37°C (but in no event to exceed 58°C) to facilitate equal agar distribution over the plate surface.

- g. Allow the plate to cool until the agar sets.

- h. Cover the TLC plate holder to prevent contamination and to prevent evaporation of moisture from the plate.

- i. Incubate the bioautograph overnight (16-18 hrs) at 37°C .

- j. Subsequent to incubation, spray the plate, using a reagent sprayer with a solution of INT (refer to section C, Reagent and Solution List, item p). Allow the color to develop for a period of approximately 2-4 hours. Additional spraying may be used to increase the rate and degree of color contrast.
-

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Estimation of
Concentration

-
- a. After incubation, locate the monensin zones on the bioautograph at the R_f of 2.5 to 3.
 - b. Estimate monensin concentration by visually comparing zone sizes from experimental samples to zone sizes from standard recovery samples.
 - i. Negative samples are reported as no activity at a sensitivity of the lowest possible standard recovery.
 - ii. Positive samples are reported as the concentration of the corresponding standard recovery level or as a range between two standard recoveries.

NOTE: Experimental samples yielding zones larger than the highest standard recovery should be diluted to yield zones within the range of the recoveries. Monensin levels obtained in this manner are regarded as approximate rather than absolute values.

- c. If permanent records of bioautographs are desired, label the plates for identification and photograph by either transmitted or reflected light.

NOTE: Polaroid photography of bioautographs has the advantage of producing a print immediately so that a satisfactory print may be obtained before destruction of the bioautograph.

- d. Attempts to quantitate residues should not be made from photographs. Estimations should be made directly from the TLC bioautographic plate.
- e. Test sensitivity when standard monensin is applied to TLC plates in methanol solution is 0.025 to 0.05 μg .
- f. Test sensitivity defined by the standard recoveries processed by the extraction procedures described is generally 0.025-0.05 μg (0.025-0.05 ppm) monensin per gram of fresh tissue. However, test sensitivity is defined by the responses obtained on each individual TLC plate and the concentrations of the recoveries used with each set of test samples.
- g. The assay must be regarded as semiquantitative and is more applicable to verifying the absence of monensin than to exact measurement of monensin levels. However, differences in zone sizes between standard recoveries can be noted and ranges in monensin concentrations can be estimated.

2. Reference

Eli Lilly, NADA 38-878V.
