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Title: Screening for Malachite Green and Crystal Violet by ELISA		
Revision: 01	Replaces: CLG-MGCV1.00	Effective: 4/08/2010

Contents

A. INTRODUCTION

Malachite green (MG) and crystal violet (CV) are triphenylmethane dyes. MG has been used as a topical fungicide and antiprotozoal agent in aquaculture. CV is also known to be effective in the treatment of fungal infections. Both compounds possess mutagenic properties and are banned for use in aquaculture. They are readily absorbed into fish tissue and metabolically reduced to leucomalachite green (LMG) and leucocrystal violet (LCV), respectively. These metabolites persist in the tissue of exposed fish.

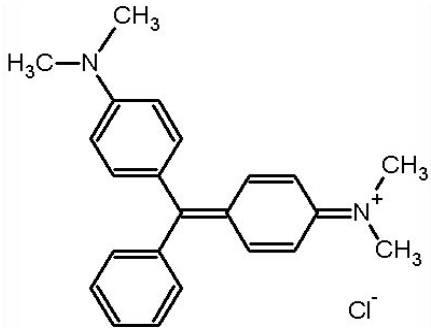
1. Theory

Malachite green, leucomalachite green, crystal violet, and leucocrystal violet are extracted from fish tissue. Leucomalachite green and leucocrystal violet are then oxidized back to malachite green and crystal violet, respectively, and detected as malachite green and crystal violet using a competitive enzyme immunoassay. The assay is carried out on microtiter plates and the resulting color intensity is measured at 450 nm in a plate reader. The assay will not differentiate between malachite green, leucomalachite green, crystal violet, and leucocrystal violet. The result will be the sum of all four compounds.

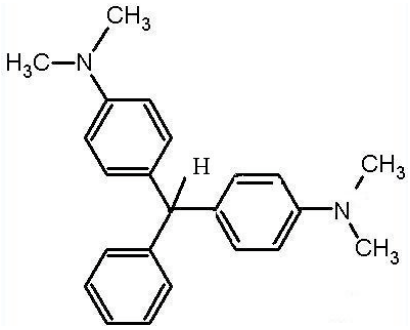
2. Applicability

This method is suitable for the analysis of malachite green, leucomalachite green, crystal violet, and leucocrystal violet in catfish at levels ≥ 1 ppb.

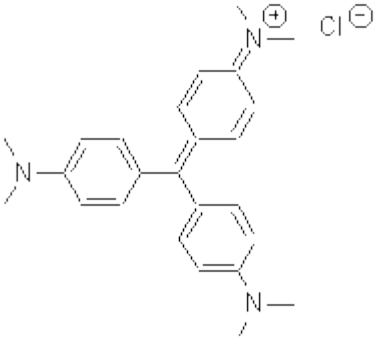
3. Structure



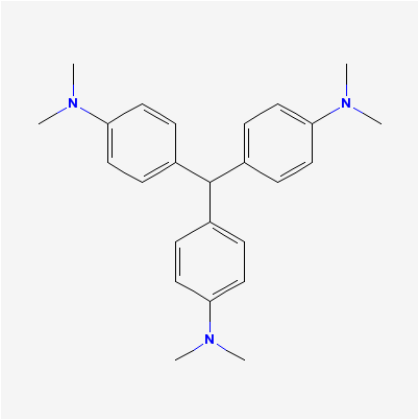
Malachite green



Leucomalachite green



Crystal violet



Leucocrystal violet

B. EQUIPMENT

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

1. Apparatus

   a. Malachite Green (MG)/LMG ELISA Test Kit-Cat. No.1019, Bioo Scientific Corp., Austin, TX.

   Note: No substitution for this kit is recommended. Kits are stored in the refrigerator at 2 - 8 °C.

   This kit contains:

   i. Malachite green antibody-coated microtiter plates (96 wells).

   ii. Malachite green standards (1.8 mL each, ready to use): 0 ppb, 0.05 ppb, 0.15 ppb, 0.5 ppb, 1.5 ppb, 4.5 ppb; 100 ppb fortification solution of malachite green (1.8 mL); 50 ppb fortification solution of leucomalachite green (1.8 mL) – upon special request see D.1.b.

   iii. 100X MG Biotin Conjugate (80 µL).

   iv. MG Biotin Conjugate Diluent (6 mL).

   v. 100X Streptavidin-HRP (200 µL).

   vi. Streptavidin-HRP Diluent (15 mL).

   vii. 20X Wash Solution (28 mL).

   viii. Stop Buffer (20 mL).

   ix. TMB Substrate (12 mL).

   x. Concentrate of Sample Extraction Buffer A (20 g containing Reagent I and II).

   xi. 10X Sample Extraction Buffer B (10 mL).

   xii. 10X Sample Extraction Buffer D (15 mL).

   xiii. 10X Oxidant Solution (1.8 mL).

   b. Centrifuge tubes - 50 mL and 15 mL with graduation, polypropylene, Falcon.

   c. Timer - Control Company.

   d. Vortex mixer - Vortex Genie, Fisher.

   e. N-EVAP - Organomation Associates, Inc.

   f. Centrifuges - Eppendorf (50 mL tubes) and VWR (15 mL tubes).

g. Pipettors - capable of dispensing volumes from 1 µL to 5 mL, Rainin and Gilson.

h. Multi-channel pipettor (50-300 µL) - Rainin.

i. Graduated cylinder - 100 mL.

j. Low actinic volumetric flasks - 100 mL, 50 mL, and 10 mL.

k. Transfer pipettes (Pasteur pipettes).

l. Glass bottles - 100 mL.

m. Dispensette - 2.5 to 25 mL (Easy calibration brand).

2. Instrumentation

a. Microplate reader - EL 808 Ultra, Bio-Tek Instruments.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted for any of the following:

1. Reagents

a. Dichloromethane - HPLC grade, Fisher.

b. Acetonitrile - HPLC grade, Acros.

c. n-Hexane - HPLC grade, Burdick and Jackson.

d. Water - HPLC grade, Fisher.

2. Solutions

Note: All solutions should be stored refrigerated at 2 - 8 °C.

a. Preparation of 1X oxidant solution:

Mix 1 volume of 10X oxidant solution with 9 volumes of acetonitrile. For example, this can be achieved by pipetting 500 µL of 10X oxidant solution into 4.5 mL acetonitrile (4.5 mL acetronitrile can be pipetted with a 5.0 mL pipettor).

b. Preparation of 1X sample extraction buffer A:

Carefully open sample extraction buffer A concentrate bag, remove reagent I and II bags and mix them in a glass bottle. Add 100 mL of distilled or HPLC grade water to dissolve the reagents.

Note: Follow example from C.2.a. to prepare solutions C.2.c. through C.2.h.

c. Preparation of 1X sample extraction buffer B:

Mix 1 volume of 10X sample extraction buffer B with 9 volumes of distilled or HPLC grade water.

d. Preparation of 1X sample extraction buffer D:

Mix 1 volume of 10X sample extraction buffer D with 9 volumes of distilled or HPLC grade water.

e. Extraction buffer D/acetonitrile solution:

Mix 4 volumes of 1X sample extraction buffer D with 1 volume of acetonitrile

(4:1 v/v).

f. Preparation of 1X wash solution:

Mix 1 volume of 20X wash solution with 19 volumes of distilled or HPLC grade water.

g. Preparation of 1X MG-biotin conjugate:

Mix 1 volume of 100X biotin conjugate with 99 volumes of biotin conjugate diluent. **IMPORTANT:** prepare fresh within 5 minutes of use and prepare only the amount needed.

h. Preparation of 1X streptavidin-HRP:

Mix 1 volume of 100X streptavidin-HRP with 99 volumes of streptavidin-HRP diluent. **IMPORTANT:** prepare fresh within 10 minutes of use and prepare only the amount needed.

i. 50:50 acetonitrile/water:

Mix 1 part acetonitrile with 1 part water. Use a graduated cylinder.

D. STANDARDS

Note: Equivalent standards may be substituted for any of the following:

1. Source

a. Leucomalachite green (Cat. No. 125660) and leucocrystal violet (Cat. No. 21915) – Sigma-Aldrich.

b. Leucomalachite green at 50 ng/mL- (special order Cat. No. 1019), Bioo Scientific.

2. Preparation

Note: Equivalent standard solutions may be prepared.

a. Stock standard solutions of leucomalachite green or leucocrystal violet at 100 µg/mL:

Weigh approximately 10 mg of leucomalachite green or leucocrystal violet into a 100 mL low actinic volumetric flask. Bring to volume with acetonitrile. Record the exact weight.

b. Intermediate standard solutions of leucomalachite green or leucocrystal violet at 1 µg/mL (prepared from stock standard solution):

Note: The amount of the stock standard solution pipetted depends on the actual weighed amount.

Use a low actinic flask. Based on the desired volume of intermediate standard, calculate the volume of stock standard solution needed to produce a 1 µg/mL intermediate standard solution. Add this volume to the flask and dilute to volume with the acetonitrile/water (50:50) mixture. For example: the standard weight is 15 mg/100 mL; pipet 6.7 µL of stock standard into a low actinic flask and add

993 µL acetonitrile/water (50:50 v/v).

c. Fortification standard solution of leucomalachite green or leucocrystal violet at 100 ng/mL:

Mix 1 volume of intermediate standard solution with 9 volumes of acetonitrile/water (50:50 v/v). For example: pipet 100 µL of intermediate standard solution into a flask and add 900 µL of acetonitrile/water (50:50 v/v); use the appropriate pipettor. Use low actinic volumetric flasks.

3. Storage and Stability

a. Malachite green and leucomalachite green standards included in the kit are stored at 2 - 8 °C and are used until the expiration date on the kit.

b. Stock standard solutions of leucomalachite green and leucocrystal violet are stored at 2 - 8 °C and are stable for 3 months.

c. Intermediate and fortification standard solutions of leucomalachite green and leucocrystal violet are stored at 2 - 8 °C and are stable for 1 month.

E. SAMPLE PREPARATION

Catfish samples must be processed long enough to produce a homogenous blend of tissues, but not long enough to become warm.

F. ANALYTICAL PROCEDURE

Note: The following steps are based on kit manufacturer instructions and may be subject to change. If any discrepancies exist, follow the current manufacturer instructions. Two recoveries are used. One is fortified with leucomalachite green and one with leucocrystal violet. Internal checks are fortified with either leucomalachite green or leucocrystal violet. Malachite green and crystal violet spikes are not necessary, because the “leuco” compounds are converted to the respective “parent” compound. Only the “parent” compound is detected by the kit.

1. Sample extraction

   a. Weigh 2.0 ± 0.1 g of test sample into a 50 mL polypropylene centrifuge tube.

   b. Weigh five 2 g portions of previously analyzed blank tissue to be used as positive (2) and negative (3) controls. Fortify one positive control with leucomalachite green and one with leucocrystal violet at 1 ppb per 20 samples.

   c. Add 0.6 mL of 1X sample extraction buffer A, 0.4 mL of 1X sample extraction buffer B and 6.5 mL of acetonitrile.

   d. Vortex for 3 minutes at maximum speed.

   e. Add 3.0 mL of water and 2.0 mL of dichloromethane.

   f. Mix gently for 1 minute.

   g. Centrifuge the samples for 5 minutes at 4000 rpm.

   h. Transfer approximately 3 mL of the upper organic layer into a graduated 15 mL polypropylene centrifuge tube containing 100 µL of 1X oxidant solution.

   i. Keep the samples at room temperature (20 - 25 °C) for 25 ± 5 minutes.

   j. Bring the samples to dryness on an N-EVAP by blowing nitrogen gas in a 50 - 60 °C water bath.

   k. Add 2 mL of n-hexane to dissolve the sample and then add 1.6 mL of extraction buffer D/acetonitrile solution.

   l. Mix the samples gently for 1 minute.

   m. Centrifuge the samples for 10 minutes at 4000 rpm.

   n. Aspirate the upper organic layer completely.

   o. Use 90 µL of the lower aqueous layer for the ELISA.

   Note: The prepared sample can be tested within 24 hours. The dilution factor is 2.0.

2. ELISA

   Note: Bring all reagents to room temperature (20 – 25 °C) before use (about 2 hours). Return all reagents to 2 - 8 °C after use. Mix all reagents by gently inverting or swirling prior to use. Prepare volumes needed for the number of microwells used. Do not allow microwells to completely dry between working steps. Do not apply more than three test strips when a single step pipette is used. Run one 0 ppb, one 0.05 ppb, one 0.15 ppb and one 0.50 ppb standard with each set to establish plate acceptability. Use the kit supplied standards.

   Insert sufficient number of wells into a microwell holder. Record standard and sample positions.

a. Pipet 90 µL of standard or extracted sample into separate wells; use a new pipette tip for each standard or sample. Pipet standards in increasing concentration. Plate the sample extracts of the 3 tissue blanks in duplicate. Plate one set of duplicates at the beginning, one set in the middle and one set towards the end of the sample set. Plate the sample extracts of the recoveries in duplicate; plate them once at the beginning and once at the end of the set. Plate the sample extract of the internal check in duplicate.

b. Pipet 30 µL of 1X MG-Biotin conjugate into each well.

c. Mix well by gently rocking the plate manually for 1 minute.

d. Incubate the plate for 30 minutes at room temperature (20 - 25 °C) in the dark (i.e. lab counter drawer).

e. Wash the plate 3 times with approximately 250 µL of 1X wash solution using a multi-channel pipettor or wash bottle. After the last wash, tap dry the plate on a paper towel. Do not allow the plate to air dry between working steps.

f. Pipet 100 µL of 1X Streptavidin-HRP into each well and mix manually by gently rocking the plate.

g. Incubate the plate for 15 minutes at room temperature (20 - 25 °C) in the dark.

h. Wash the plate 3 times with approximately 250 µL of 1X wash solution using a multi-channel pipettor or wash bottle. After the last wash tap dry the plate on a paper towel. Do not allow the plate to air dry between working steps.

i. Pipet 100 µL of TMB substrate into each well. Any substrate solution showing discoloration is indicative of deterioration and should be discarded.

j. Time the reaction immediately after adding the substrate. Mix the solution by gently rocking the plate manually for 1 minute while incubating. Incubate for 15 ± 1 minutes at room temperature (20 - 25 °C) in the dark.

k. Pipet 100 µL of stop buffer into each well.

l. Measure the absorbance at 450 nm against an air blank. Read within 10 minutes.

3. Instrumental settings

Use a plate reader set at 450 nm for evaluation.

G. CALCULATIONS

Average the UV absorbance readings of the recoveries and internal check wells since they are run in duplicate.

Calculate the average (AVG), standard deviation (SD) and coefficient of variation (CV) for the six tissue blank UV absorbance readings. Calculate the decision level (DL) as follows:

Decision level (DL): Average tissue blank UV absorbance reading – 3 x standard deviation.

DL= AVG_{Blank} – 3 x SD

Any sample having an UV absorbance reading of ≤ DL is considered positive.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment — Wear gloves, laboratory coat and safety glasses.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Dichloromethane	Vapors are harmful to skin, eyes, and respiratory system. Potential carcinogen and teratogen.	Work in fume hood.
n-Hexane	Flammable. Harmful if swallowed or inhaled. Causes respiratory, eye, and skin irritation.	Work in fume hood. Keep away from flame or heat.
Acetonitrile	Flammable. Harmful if swallowed or inhaled. Causes respiratory, eye, and skin irritation.	Work in fume hood. Keep away from flame or heat.
Leucomalachite green	Harmful if inhaled or ingested. Causes eye, skin, and respiratory irritation.	Work in fume hood.
Leucocrystal violet	Harmful if inhaled or ingested. Causes eye, skin, and respiratory irritation.	Work in fume hood.

3. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Dichloromethane	See above	Collect waste in tightly sealed container. Store away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet. Dispose of in accordance with local, state, and federal regulations.
n-Hexane	See above	Collect waste in tightly sealed container. Store away from non-compatibles in a cool, well ventilated,

		flammable liquid storage area/cabinet. Dispose of in accordance with local, state, and federal regulations.
Acetonitrile	See above	Collect waste in tightly sealed container. Store away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet. Dispose of in accordance with local, state, and federal regulations.
Leucomalachite green	See above	Collect waste in tightly sealed container. Dispose of in accordance with local, state, and federal regulations.
Leucocrystal violet	See above	Collect waste in tightly sealed container. Dispose of in accordance with local, state, and federal regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standard
   a. A plate must meet all of the following criteria:
      i. No false negatives for the 1 ppb recoveries or false positives for the negative controls
      ii. The absorbances must decrease from the 0 ppb to the 0.50 ppb standard wells.
      iii. A variability in absorbance of less than ± 25% between duplicate measurements of the recoveries and internal check is acceptable. This is determined as follows: $|x-y|/ [(x+y)/2] * 100$
      iv. The coefficient of variation (CV) of the six tissue blank measurements has to be ≤ 20%.

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	Incubation time (F.2.j.)	15 minutes ± 1 minute
b.	TMB substrate (F.2.i.)	Discard if discolored
c.	Incubation time (F.1.i.)	25 minutes ± 5 minutes
d.	Incubation temperature (F.2.)	20 – 25 °C

3. Readiness To Perform

   a. Familiarization

      i. Phase I: Standards on each of 3 consecutive days, which will include the following:

         (a) 0 ppb

         (b) 0.05 ppb

         (c) 0.15 ppb

         (d) 0.50 ppb

      ii. Phase II: Fortified samples-3 replicates at 1 ppb over a period of 3 different days for leucomalachite green and leucocrystal violet.

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

      iii. Phase III: Check samples for analyst accreditation.

         (a) 8 unknown samples. The fortifications, including the number of blanks, are to be blind to the analyst. At least 2 of the 8 should be blank, 3 must be fortified at 1 ppb leucomalachite green, and 3 must be fortified at 1 ppb leucocrystal violet.

         (b) Report analytical findings to the Laboratory Quality Assurance Manager (QAM) and/or Supervisor.

         (c) Authorization from QAM and Supervisor is required to commence official analysis.

   b. Acceptability criteria.

      Refer to I. 1.

4. Intralaboratory Check Samples

   a. System, minimum contents.

      i. Frequency: 1 check sample per week for each analyst when samples are analyzed. The check sample can be either leucomalachite green or leucocrystal violet.

      ii. Records are to be maintained by the analyst.

   b. Acceptability criteria.

      Refer to I. 1.

      If unacceptable values are obtained, then:

      i. Stop all official analyses by the analyst for this method.

      ii. Take corrective action.

5. Sample Acceptability and Stability
   a. Matrix: catfish tissue, mainly muscle but can include other parts (skin, bones).
   b. Condition upon receipt: cold, unspoiled and sealed from air.
   c. Sample storage:
      i. Time and condition: 24 months frozen; avoid repeated thaw/freeze cycles.

6. Sample Set
   a. Each sample set must contain:
      i. standards at 0, 0.05, 0.15, and 0.50 ppb
      ii. positive control for leucomalachite green at 1 ppb
      iii. positive control for leucocrystal violet at 1 ppb
      iv. 3 blank tissue controls
      v. samples

7. Analyst Capability
   a. Minimum proficiency level (MPL): 1 ppb

J. WORKSHEET

[RESERVED]

K. APPENDIX

1. References
   a. Test kit instructions Malachite Green/LMG, Bioo Scientific
   b. Confirmatory analysis of malachite green, leucomalachite green, crystal violet and leucocrystal violet in salmon by liquid chromatography-tandem mass spectrometry. Geraldine Dowling, Patrick P.J. Mulder, Conor Duffy, Liam Regan, Malcolm R. Smyth, Analytica Chimica Acta **586**, (2007), 411-419 --for standard stability information.

L. APPROVALS AND AUTHORITIES

1. Approvals on file.

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