CLG-MG/CV2.00		Page 1 of 15
Title: Confirmation of Malachite Green and Crystal Violet by UHPLC-MS-MS		
Revision: Original	Replaces: NA	Effective: 10/13/2010

Contents

A. INTRODUCTION

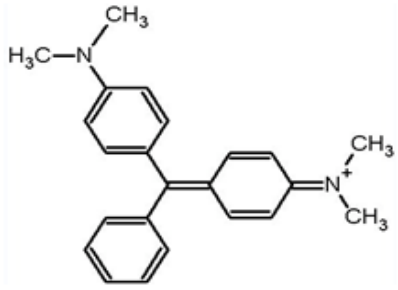
1. Background

Malachite green (MG), crystal violet (CV), leucomalachite green (LMG), and leucocrystal violet (LCV) are extracted from catfish tissue using a McIlvaine buffer-acetontrile solvent combination. The resulting organic extract is further purified using neutral alumina and a cation exchange solid phase extraction system. After evaporating the eluate to dryness, it is reconstituted in a mixture of methanol and acetate buffer and analyzed by ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS-MS).

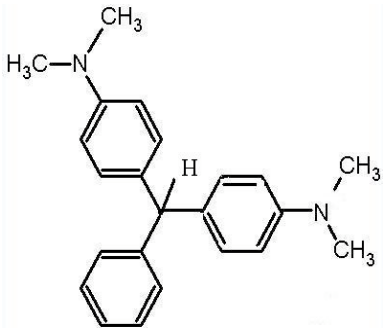
2. Applicability

This method is applicable for the confirmation of MG, CV, LMG, and LCV in catfish tissue at levels ≥ 1 ppb.

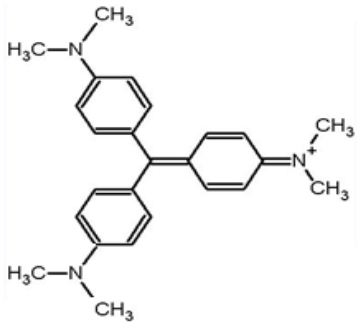
3. Structure



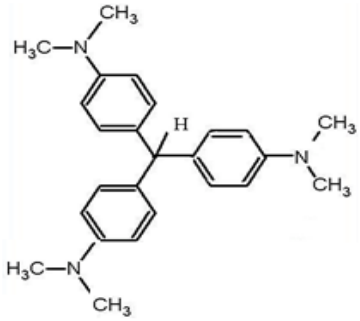
Malachite green



Leucomalachite green



Crystal violet



Leucocrystal violet

B. EQUIPMENT

Note: Equivalent apparatus and instrumentation may be substituted.

1. Apparatus
   a. Balance – Cat. No. XS2002S, Mettler.
   b. Analytical balance – Cat. No. AE163, Mettler.
   c. Centrifuge – Cat. No. 5810, Eppendorf.
   d. Vortex mixer – Fisher Genie 2.
   e. N-EVAP – Organomation 111.
   f. 50 mL polypropylene tubes – Cat. No. 352070, Falcon.
   g. 15 mL polypropylene tubes – Cat. No. 352097, Falcon.

h. Pipettes capable of dispensing 5 – 5000 µL – Rainin.

i. Pasteur Pipette – Cat. No. 14673-010, VWR.

j. Amber LC Vials – Cat. No. EP339-20-ACT, VWR.

2. Instrumentation

a. UPLC-MS-MS

i. Liquid Chromatograph – Acquity UPLC, Waters Corp.

ii. Analytical Column – Acquity UPLC, C18 1.7 µm, 2.1 x 50 mm, Waters Corp.

iii. Mass Spectrometer – Acquity TQD, Waters Corp.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted.

1. Reagents

a. L-Ascorbic acid – Cat. No. A5960, Sigma-Aldrich.

b. Sodium hydrogen phosphate (Na_2HPO_4) – Cat. No. 11643, Sigma-Aldrich.

c. *p*-Toluenesulfonic acid (*p*-TSA) hydrate – Cat. No. 139020050, Acros.

d. Ammonium hydroxide – Cat. No. A470, Fisher.

e. Methanol – Cat. No. A-454, Fisher.

f. Acetic acid – Cat. No. AX0074-6, EMD.

g. Acetonitrile – Cat. No. A-996, Fisher.

h. Ammonium acetate – Cat. No. 0596-01, Baker.

i. Water – Cat. No. W7, Fisher.

j. N,N,N',N'-Tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) – Cat. No. T3134, Sigma-Aldrich.

k. Alumina – Cat. No. A950, Fisher.

l. Sodium Chloride – Cat. No. S642, Fisher.

m. Citric acid – Cat. No. 251275, Sigma-Aldrich.

n. SPE cartridges – Oasis MCX, 6 mL, 150 mg sorbent, Waters.

2. Solutions

a. 0.2 M sodium hydrogen phosphate:

Dissolve 35.6 g in water and bring to a total volume of 1 L.

b. 0. 02 M ammonium acetate:

Dissolve 1.54 g in water and bring to a total volume of 1 L. Adjust to pH 4 with glacial acetic acid.

c. 1 M *p*-TSA (*p*-Toluenesulfonic acid):

Dissolve 19 g p-TSA hydrate in water and bring to a total volume of 100 mL.

d. 0.1 M citric acid:

Dissolve 19.2 g citric acid in water and bring to a total volume of 1 L.

e. 0.5 mg/mL L-ascorbic acid solution:

Dissolve 50 mg L-ascorbic acid in 5 mL methanol and 95 mL acetonitrile.

f. SPE eluting solvent:

Mix 5 mL ammonium hydroxide with 5 mL 0.5 mg/mL L-ascorbic acid solution (C.2.e) and 90 mL acetonitrile.

g. McIlvaine buffer pH 3.0:

Mix 100 mL 0.2 M sodium hydrogen phosphate (C.2.a) with 430 mL 0.1 M citric acid (C.2.d).

h. Sample and LC standard dilution solvent:

Mix 70 mL methanol with 30 mL 0.02 M ammonium acetate (pH 4) (C.2.b).

i. TMPD (1 mg/mL):

Dissolve 10 mg TMPD in 10 mL methanol.

D. STANDARDS

1. Source

a. Malachite green oxalate salt (MG) – Cat. No. M6880, Sigma-Aldrich.

b. Crystal violet chloride salt (CV) – Cat. No. G2039, Sigma-Aldrich.

c. Leucomalachite green (LMG) – Cat. No. 125660, Sigma-Aldrich.

d. Leucocrystal violet (LCV) – Cat. No. 219215, Sigma-Aldrich.

2. Preparation

a. MG, LMG, CV, LCV Stock solutions (~1 mg/mL):

Add 28.4 ± 5 mg MG oxalate, 20 ± 5 mg LMG, 22 ± 5 mg CV chloride and 20 ± 5 mg LCV into separate amber vials. Dissolve in 20 mL acetonitrile. Calculate the exact concentration of the stock solutions taking the actual weight and purity into account.

These solutions are to be stored refrigerated (2 – 8 °C) and expire in 6 months.

b. Combined working solution (1.0 µg/mL):

Pipet ~10 µL (depending on the exact concentration) of each stock standard (D.2.a) into a 10.0 mL amber volumetric flask and bring to volume with acetonitrile.

This solution is to be stored refrigerated (2 – 8 °C) and expires in 1 month.

c. External Standard (5.0 ng/mL):

Pipet 5 µL of combined working solution (D.2.b) into 995 µL standard dilution solvent in an amber LC vial.

This solution is to be prepared fresh for each sample set.

E. SAMPLE PREPARATION

Catfish tissue must be processed long enough to produce a homogeneous blend of tissue, but not long enough to become warm.

F. ANALYTICAL PROCEDURE

1. Sample extraction

a. Weigh 5.0 ± 0.2 g catfish samples into 50 mL polypropylene centrifuge tubes.

b. Weigh two 5.0 ± 0.2 g blank catfish samples to be used for negative (blank) and positive (fortified) controls.

c. Fortify the positive control with 5 µL of the combined working standard solution (D.2.b), which is equivalent to 5 ng of each analyte. Vortex for about 30 seconds and allow to stand in the dark for 10 minutes.

d. To all samples, add 4 mL McIlvaine buffer, 100 µL 1 mg/mL TMPD and 100 µL 1 M *p*-TSA.

e. Vortex for about 30 seconds.

f. Add 25 mL acetonitrile and vortex about 1 minute.

g. Add 5 g NaCl, vortex about 20 seconds and centrifuge at 4000 RPM for 5 min.

h. SPE Column Clean-up:

i. Prepare the SPE columns by adding 2 g alumina above the MCX sorbent bed to form a second bed.

ii. Wash the columns quickly with two 3 - 5 mL portions of acetonitrile to allow air bubbles to escape.

iii. Load the columns with the top layer in the centrifuge tubes from step g.

iv. Wash each column with approximately 5 mL acetonitrile.

v. Remove all of the alumina from the SPE column by forcefully filling the column with acetonitrile and quickly inverting. This may need to be repeated once or twice to ensure all the alumina is gone.

vi. Elute the columns into 15 mL polypropylene tubes by filling with the SPE elution solvent.

i. Evaporate each tube to dryness at < 50 °C in an N-Evap under a gentle nitrogen stream.

j. Add 1 mL of 7:3 (v/v) methanol:ammonium acetate buffer (C.2.b.) to the residue and vortex briefly.

k. Transfer each extract to an amber LC vial.

2. UPLC-MS-MS analysis

Note: The following instrument parameters may be optimized.

a. UPLC Instrumental Settings

Column temperature: 40 °C
Injection volume: 5 µL

UPLC Time Program:

Time (min)	Flow Rate (mL/min)	(A) 0.02M ammonium acetate (pH 4) (%)	(B) Acetonitrile (%)
0	0.3	70	30
1	0.3	50	50
2	0.3	10	90
4	0.3	10	90
4.1	0.3	70	30
8	0.3	70	30

b. MS/MS settings

Note: Tune the instrument as needed.

Ion Mode: ESI +
Source Temperature: 150 ºC

Desolvation Temperature: 350 ºC
Dwell Time (s): 0.05
Capillary voltage: 3 kV

Summary of Multiple Reaction Monitoring (MRM) transitions and parameters selected for each compound:

Compound	RT (min)	Precursor Ion (m/z)	Cone (V)	Product ions (m/z)	Collision (eV)
CV	2.1	372.2	70	340.4	55
			70	356.4	45
MG	1.8	329.2	70	208.2	35
			70	313.3	35
LCV	2.9	374.2	50	238.3	30
			50	358.3	22
LMG	2.9	331.2	50	316.3	30
			50	239.3	30

G. CONFIRMATION

a. Retention time of the sample must be ± 5% of the external standard or positive control.

b. All monitored product ions must exhibit a S/N ratio > 3.

c. Product ion ratio of all samples must differ from the external standard or positive control by no more than 10% absolute difference.

d. For positive samples, the peak area must be at least 10% of the external standard or positive control area for the most abundant ion.

Note: An external standard or positive control is injected at the beginning and end of each set. Comparisons are relative to the average of the two injections or the first injection alone.

H. SAFETY INFORMATION AND PRECAUTIONS

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

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Malachite green	Harmful if ingested. Risk of serious damage to eye. Potential teratogen.	Work in fume hood.
Leucomalachite green	Harmful if inhaled or ingested. Causes eye, skin, and respiratory irritation.	Work in fume hood.
Crystal violet	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.
Acetonitrile	Flammable. Harmful if swallowed or inhaled. Causes respiratory, eye, and skin irritation.	Work in fume hood. Keep away from flame or heat.
Methanol	Flammable. Vapors are corrosive to the skin, eyes, and respiratory system.	Avoid contact or prolonged exposure to vapors. Work in a fume hood. Keep away from flame or heat.

3. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Malachite green	See above	Collect waste in tightly sealed container. 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.

Crystal violet	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.
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.
Methanol	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.

I. QUALITY ASSURANCE PLAN

1. Performance Standard
   a. All ions must be present in the positive control.
   b. Negative control (blank) must be no greater than 10% of the external standard or positive control area for each analyte.
2. Critical Control Points and Specifications

   There are no known critical control points.

3. Readiness To Perform
   a. Familiarization
      i. Phase I: Standard solutions of all four analytes at 5 ng/mL (equivalent to 1 ppb based on positive control) on each of three days.
      ii. Phase II: Fortified samples-3 replicates of all four analytes at 1 ng/g (1 ppb) over a period of 3 days. Include a blank sample with each set.

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

iii. Phase III: Check samples for analyst accreditation.

(a) 8 samples unknown to the analyst. At least one should be blank and the others fortified at 1 ng/g (1 ppb).

(b) Report analytical findings to 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: One check sample per week when samples are analyzed.

ii. Records are to be maintained by the analyst.

a. Acceptability criteria.

Refer to I.1.

If unacceptable values are obtained, then:

i. Stop all official analyses by that 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: 24 months.

ii. Condition: Frozen.

6. Sample Set

a. Each set must contain:

i. External Standard.

ii. Positive control (spiked tissue).

iii. Negative control (blank tissue).

iv. Samples.

7. Analyst Capability

   Minimum Level of Applicability (MLA): 1 ng/g (1 ppb)

J. WORKSHEET

Malachite Green/Crystal Violet Confirmation

Date Started: **Analyst:**

Date completed: **Reviewer:**

#	ILN #	Sample Weight 5 g	Spiking Amount (ppb)	Result (pos/neg)				Comments
				MG	CV	LMG	LCV	
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								

Equipment	ILN	Reagents	ILN
Balance		p-TSA	
Pipettor		TMPD	
Pipettor		SPE elution solution	
Pipettor		McIlvaine buffer	
N-EVAP		Acetonitrile	
Centrifuge		Sample/Std diluent	
LC/MS		SPE lot number	
		Sodium Chloride	

Spiking solution-ILN_____

QC matrix_____

K. APPENDIX

1. References

Chen, Guoying and Miao, Shui. “HPLC Determination and MS Confirmation of Malachite Green, Gentian Violet, and Their Leuco Metabolite Residues in Channel Catfish Muscle”, USDA, ARS, ERRC, Wyndmoor, PA and Shanghai Institute for Food and Drug Control, Shanghai, China.

1. Chromatograms

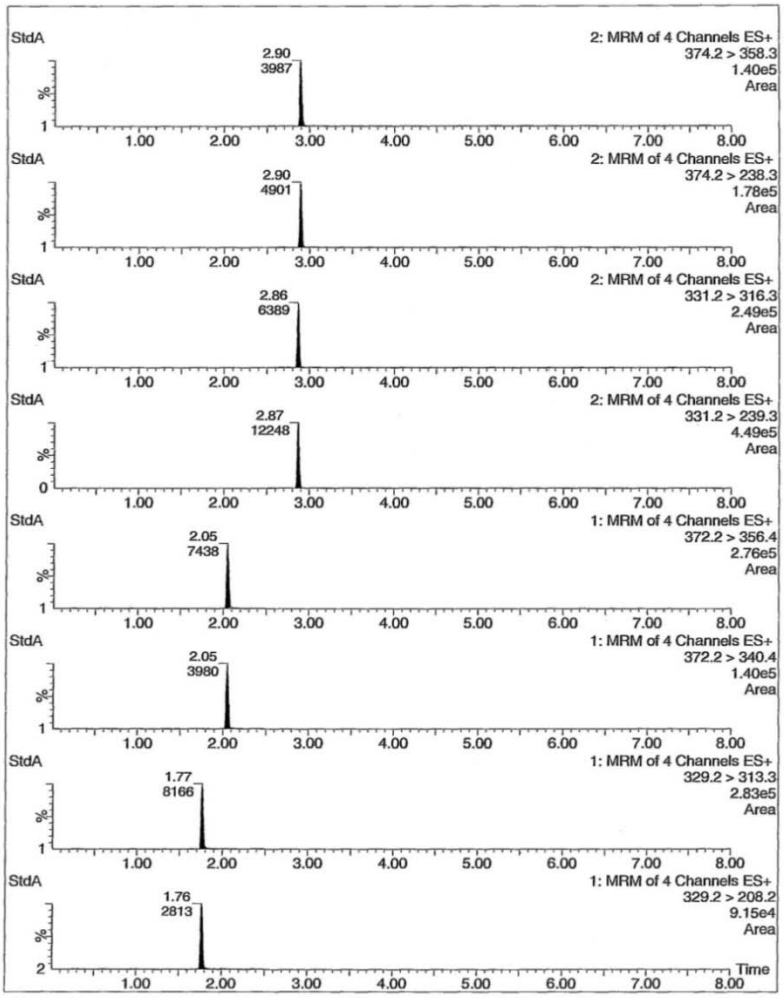


Figure 1. MRM Transitions of Mixed External Standards of MG, CV, LCV & LMG at 1 ppb level.

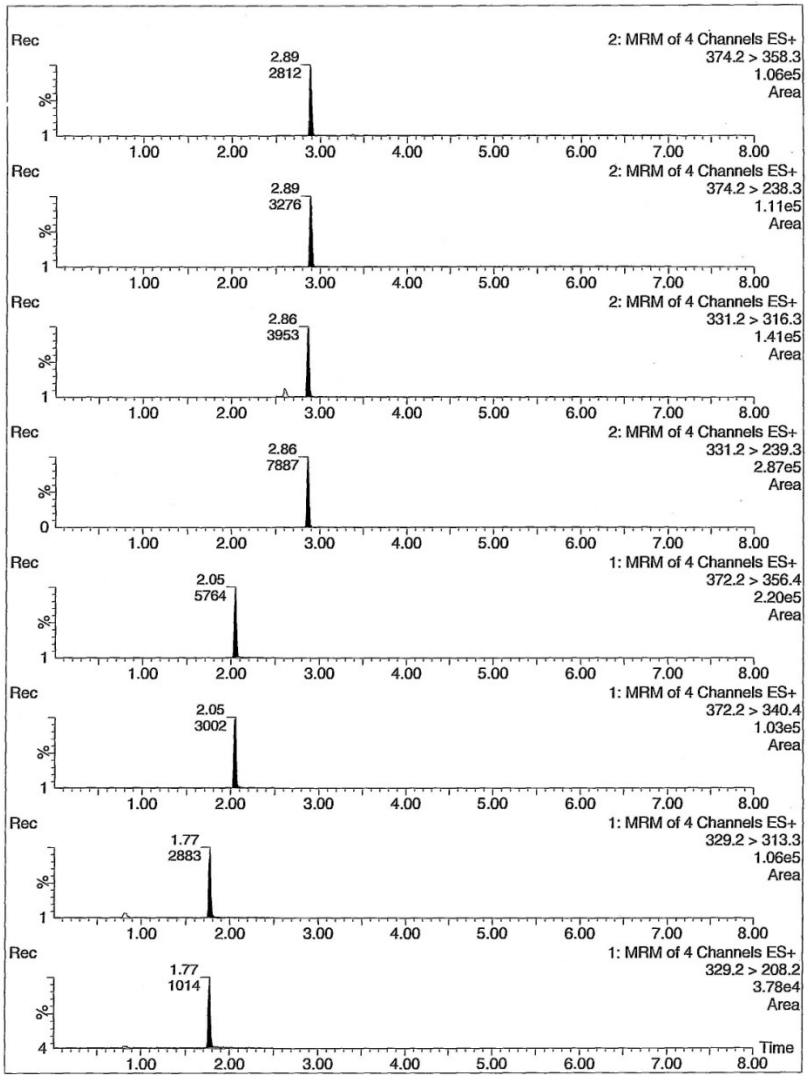


Figure 2. MRM Transitions of a Positive Control Spiked with a Mixed Standard of MG, CV, LCV & LMG at 1 ppb level.

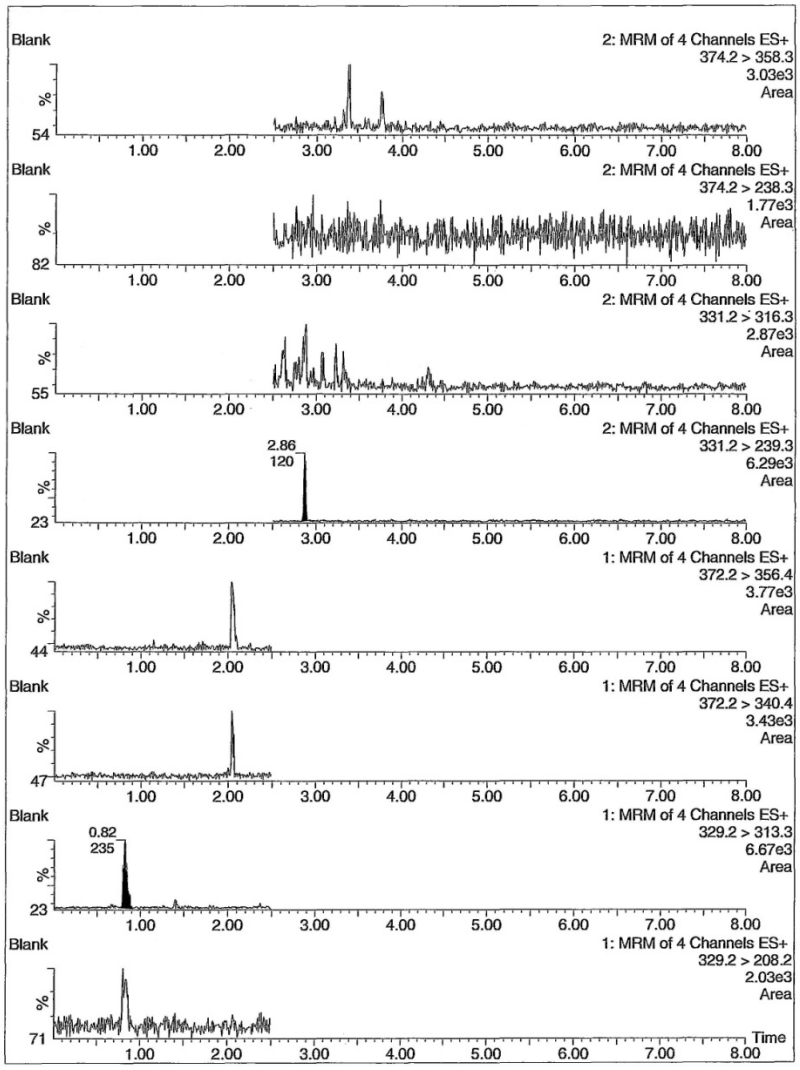


Figure 3. MRM Transitions of Blank Catfish Muscle

L. APPROVALS AND AUTHORITIES

1. Approvals on file.

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