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Title: Confirmation of Malachite Green and Crystal Violet by UHPLC-MS-MS		
Revision: .01	Replaces: MGCV 2.00	Effective: 06/20/2016

Contents

A. INTRODUCTION

1. Summary of Procedure

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 fish of the order Siluriformes (catfish) tissue at levels ≥ 1 ppb.

Note: Refer to 21CFR for tolerance values set by FDA and 40CFR for tolerance values set by EPA

B. EQUIPMENT

Note: Equivalent equipment 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 / solutions may be substituted. The stability time frame of the solution is dependant on the expiration dates of the compounds used. The maximum length of time that a working reagent shall be used is 1 year unless the laboratory has produced extension data.

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

Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations. The stability time frame of the solution is dependant on the expiration date of the components used. In-house prepared standards shall be assigned an expiration date that is no later than the expiration date of the earlilest expiring component or no later than the stability stated in the method, whichever ends soonest. The maximum length of time that an in-house prepared standard shall be used is 1 year unless the laboratory has produced extension data.

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. Preparation of Controls and Samples

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

   b. Weigh out 5.0 ± 0.2 g portions of a known blank catfish tissue into a 50 mL polypropylene centrifuge tube for each of the following Quality Control samples as needed:

      i. A tissue blank (negative control) - one needed for each analytical batch.

      ii. Positive Control - Fortify the control sample with 5 µL of the 1.0 µg /mL Combined Working 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.

      iii. An internal check sample (as needed).

2. Extraction Procedure

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

   b. Vortex for about 30 seconds.

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

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

   e. 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.

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

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

   h. Transfer each extract to an amber LC vial.

3. Instrumental Settings

   UPLC-MS-MS analysis

   Note: The instrument parameters may be optimized to ensure system suitability.

   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

4. Sample Set
   a. For each batch of up to 20 samples, include:
      i. External Standard.
      ii. Positive control (spiked tissue).
      iii. Negative control (blank tissue).
      iv. Check Sample (if needed)
      v. Samples

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.	Work in fume hood.

	Causes eye, skin, and respiratory irritation.	
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

   Follow federal, state and local 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. Intralaboratory Check Samples
   a. System, minimum contents.
      i. Frequency: One per week per analyst when samples analyzed.
      ii. Records are to be maintained.
   b. Acceptability criteria.

      Refer to I.1.

      If unacceptable values are obtained, then:

      i. Investigate following established procedures.

ii. Take corrective action as warranted.

4. Sample Condition upon Receipt: cold, unspoiled, and sealed from air.

J. 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.

2. Chromatograms

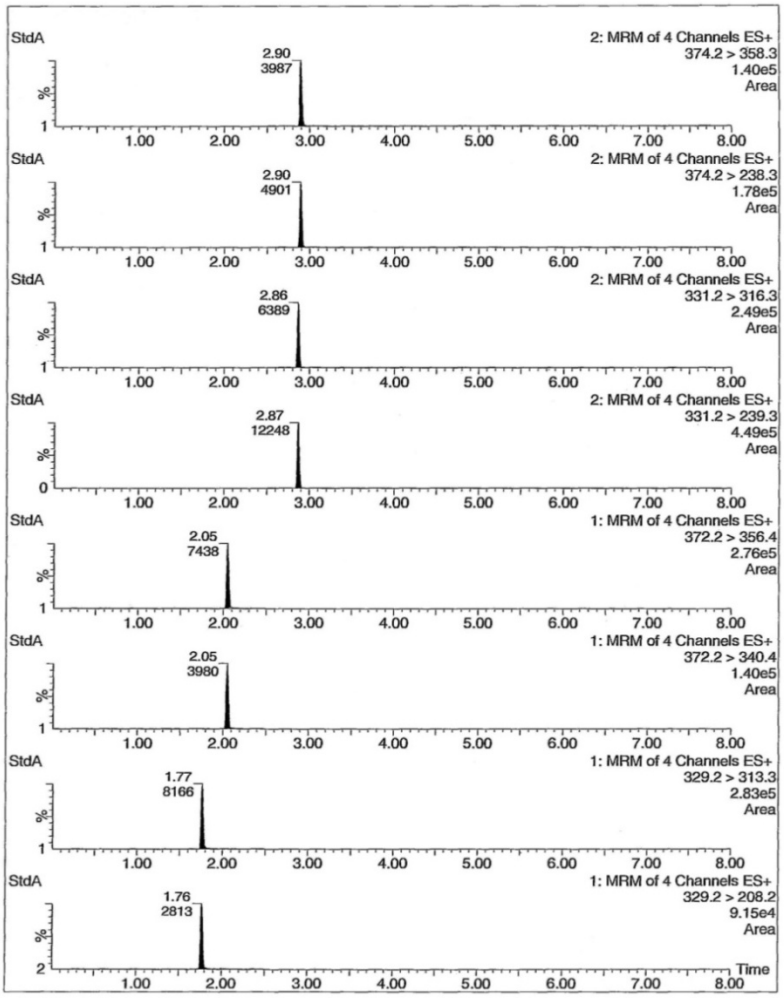


Figure 1. MRM Transitions of Mixed External Standards of MG, CV, LCV & LMG at 1 ppb level.

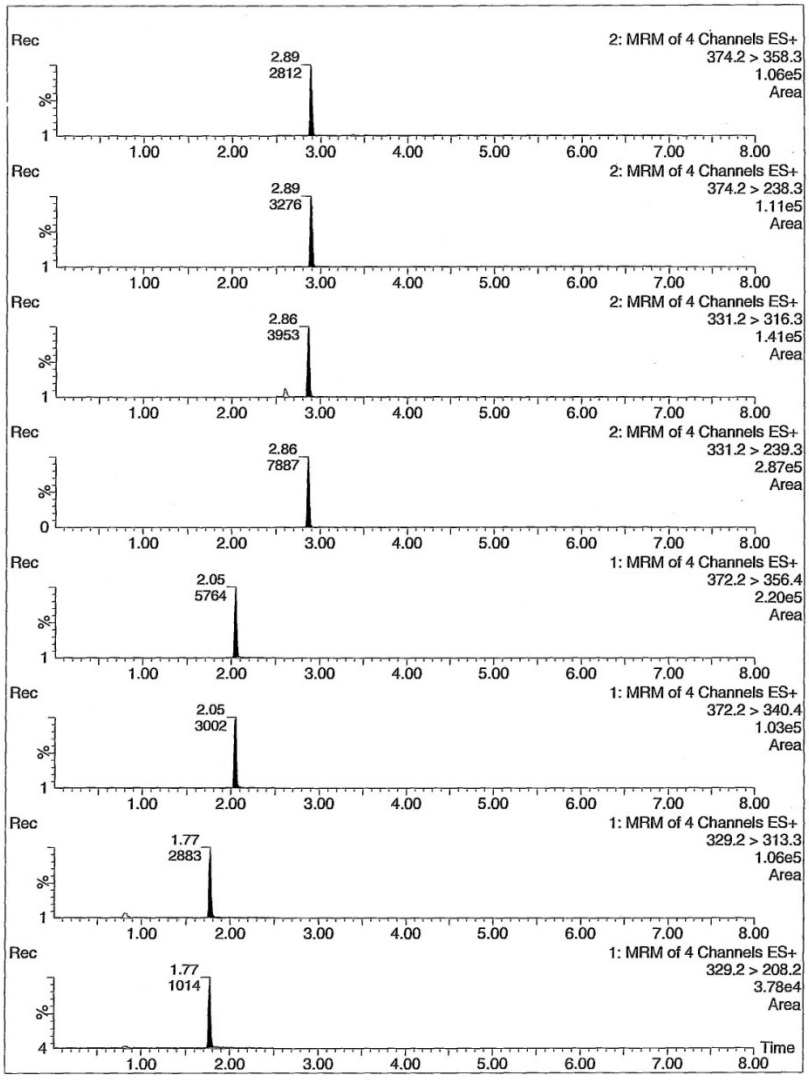


Figure 2. MRM Transitions of a Positive Control Spiked with a Mixed Standard of MG, CV, LCV & LMG at 1 ppb level.

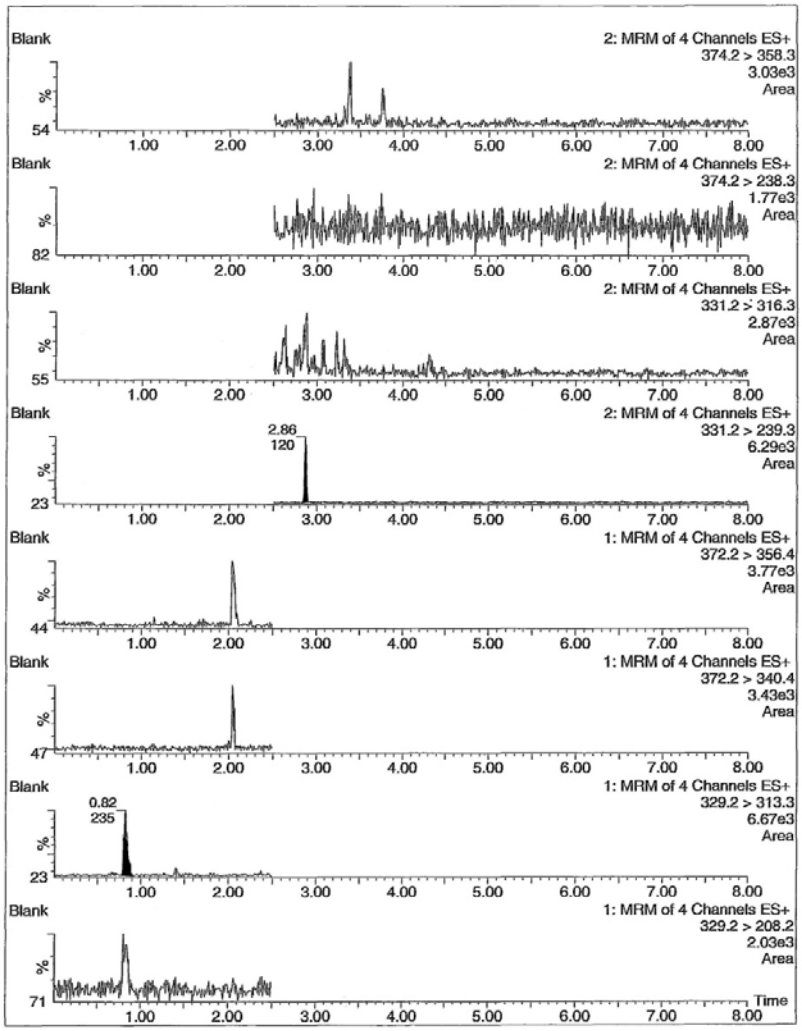
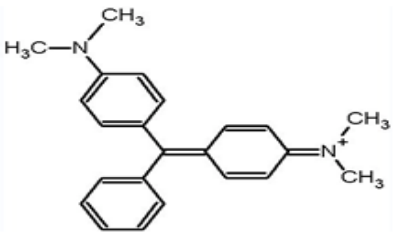
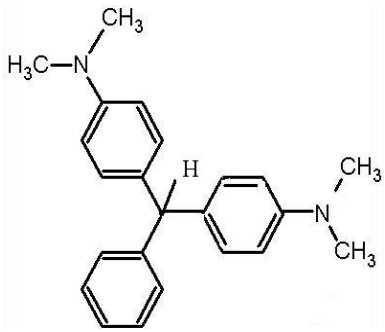


Figure 3. MRM Transitions of Blank Catfish Muscle

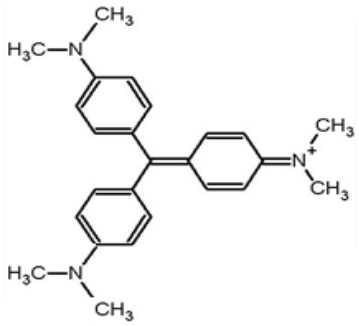
3. Structure



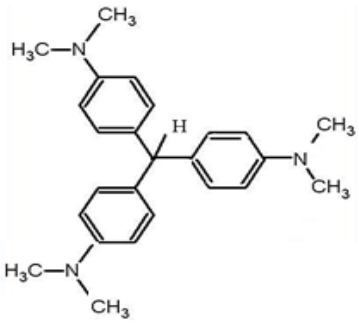
Malachite green



Leucomalachite green



Crystal violet



Leucocrystal violet

4. Minimum Level Of Applicability(MLA)

   1 ng/g (1 ppb)

K. APPROVALS AND AUTHORITIES

1. Approvals on file.

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