

**United States Department of Agriculture  
Food Safety and Inspection Service, Office of Public Health Science**

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Title: Determination and Confirmation of Malachite Green and Crystal violet by UHPLC-MS-MS		
Revision: Original	Replaces: NA	Effective: 05/12/2019

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**A. INTRODUCTION**

1. Summary of Procedure

Triphenylmethane dyes and their leuco metabolites, in the presence of hydroxylamine and anhydrous magnesium sulfate, are extracted from catfish tissue with acetonitrile. After evaporation of the extract, the residue is redissolved in acetonitrile/ascorbic acid and then analyzed using LC-MS/MS. Quantitative analysis uses extracted matrix calibrants and four isotopically labeled internal standards to correct for matrix effects and extraction losses.

2. Applicability

This method is suitable for the quantification and/or confirmation of MG (Malachite Green), CV (Crystal Violet), LMG (LeucoMalachite Green), and LCV (LeucoCrystal Violet) in fish of the order Siluriformes (catfish) tissue at levels  $\geq 1$  ppb.

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

Note: This method may be performed using standards/solutions that contain fewer analytes than the method is applicable for, if the excluded analytes will not be included in the reported results.

**B. EQUIPMENT**

Note: Equivalent equipment may be substituted.

1. Apparatus:

- a. Balance - Cat. No. XS2002S, Mettler.
- b. Analytical balance – Cat No. AE 163, Mettler
- c. Vortex mixer
- d. Multitube vortexer (platform shaker)
- e. Centrifuge and tubes — Capable of accelerating 50 mL polypropylene centrifuge tubes (or equivalent) to  $2000 \times g$  and refrigerated to  $4^{\circ}\text{C}$ .
- f. Transfer pipets — Disposable
- g. Nitrogen evaporator — Capable of heating sample tubes to  $50^{\circ}\text{C}$ .
- h. Evaporation tubes — 10–15 mL polypropylene tubes, or equivalent.
- i. Micro centrifuge and tubes — Capable of accelerating micro centrifuge tubes containing 800  $\mu\text{L}$  of volume to  $20\,000 \times g$ .
- j. PVDF syringe filters and syringes — 0.45  $\mu\text{m}$ , 13 mm Millex-HV EMD, Millipore Corp., Billerica, MA, USA and 1 mL disposable syringes, or equivalent

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- k. Autosampler vials — Glass or polypropylene, with caps. Amber-colored vials recommended to protect light sensitive compounds.
- l. LC column — C<sub>18</sub> stationary phase (100 × 2.1 mm, 3.5 μm) with C<sub>18</sub> guard column (optional, 10 × 2.1 mm; Waters Corp. Symmetry), or equivalent.

2. Instrumentation

LC-MS/MS System: Triple quadrupole mass spectrometer system equipped with an electrospray ionization source for operation in the positive ion mode and capable of selected reaction monitoring (SRM) with at least two transitions/analyte and one transition/internal standard (Waters Acuity, Agilent 6490, or equivalent.)

**C. REAGENTS AND SOLUTIONS**

Note: Equivalent reagents / solutions may be substituted. 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. Acetonitrile (MeCN) – HPLC grade, Sigma-Aldrich, Cat. No. AX0145.
- b. Hydroxylamine hydrochloride (NH<sub>2</sub>OH·HCl) – ACS Reagent grade, Sigma-Aldrich, Cat. No. 255580.
- c. Magnesium sulfate, anhydrous – Analytical grade, Sigma-Aldrich, Cat. No. 106067.
- d. Ammonium formate (HCOONH<sub>4</sub>) – For Mass Spectrometry, Sigma-Aldrich, Cat. No. 70221.
- e. L-Ascorbic acid – ACS Reagent grade, Sigma-Aldrich, Cat. No. 795437.
- f. Formic acid (HCOOH) – ACS Reagent grade, Sigma-Aldrich, Cat. No. 399388.
- g. Water — Milli-Q

2. Solutions

- a. 9.5 g/L Hydroxylamine solution - Dissolve 5.0±0.05 g hydroxylamine hydrochloride in Milli-Q water and dilute to a final volume of 250 mL.
- b. 1.0 g/L Ascorbic acid solution - Dissolve 100±5 mg ascorbic acid in Milli-Q water and dilute to a final volume of 100 mL.
- c. Reconstitution solution - Transfer 1.0±0.05 mL Ascorbic acid solution to approximately 90 mL of acetonitrile and dilute to a final volume of 100 mL with acetonitrile.
- d. Formic acid solution in water (5%, v/v) - Add 5 mL concentrated formic acid to approximately 90 mL Milli-Q water and dilute with Milli-Q water to a final volume of 100 mL.

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- e. 0.05 M Ammonium formate buffer - Dissolve 3.15±0.005 g of ammonium formate in approximately 900 mL Milli-Q water. Add 5 mL of the formic acid solution and dilute with Milli-Q water to a final volume of 1000 mL

**D. STANDARD(S)**

Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations. In-house prepared standards shall be assigned an expiration date that is no later than the stability stated in the method. 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. Standard Information

- a. Malachite Green oxalate salt (MG): CAS No. 2437-29-8, Sigma-Aldrich Cat. No. 46396.
- b. LeucoMalachite Green (LMG): CAS No.129-73-7, Sigma-Aldrich Cat. No. 69669.
- c. Crystal Violet (CV): CAS No. 548-62-9, Sigma-Aldrich Cat. No. 46364.
- d. LeucoCrystal Violet (LCV): CAS No. 603-48-5, Sigma-Aldrich Cat. No. 219215.
- e. Malachite Green-d5 Picrate (MG-d5): CAS No.1258668-21-1, Sigma-Aldrich Cat. No. 33945.
- f. LeucoMalachite Green-d5 (LMG-d5): CAS No. 947601-82-3, Sigma-Aldrich Cat. No. 34182.
- g. Crystal Violet-d6 trihydrate (CV-d6): CAS No. N/A, Sigma-Aldrich Cat. No. 32853.
- h. LeucoCrystal Violet-d6 (LCV-d6): CAS No. 1173023-92-1, Sigma-Aldrich Cat. No. 32634.

2. Preparation of Standard Solution(s)

a. Stock Standard solutions

Prepare individual Stock Standard solutions of each dye, metabolite, and internal standard compound. Final concentration of each compound will be ~100 µg/mL in acetonitrile. Care should be exercised to account for water presence, compound purity and any counterions that might be present in individual standards. Store all solutions in glass at -20°C and protect from light (stability = 1 year).

b. Mixed Intermediate Standard solution

The Mixed Intermediate Standard solution will be 1.000 µg/mL of each of the compounds MG, LMG, CV and LCV. To prepare combine ~1 mL of each individual stock solution in order to produce a mixture of 1.000 µg/mL of each of the analytes and dilute the final mixture to 100 mL in Class A volumetric glassware using acetonitrile. These solutions are stored in glass at -20°C and protected from light (stability = 1 month).

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c. Mixed Intermediate Internal Standard solution

The Mixed Intermediate Internal Standard solution will be 1.000 µg/mL of each of the compounds MG-d<sub>5</sub>, LMG-d<sub>5</sub>, CV-d<sub>6</sub>, and LCV-d<sub>6</sub>. To prepare combine ~1 mL of each individual stock solution and dilute the final mixture to 100 mL in Class A volumetric glassware using acetonitrile. These solutions are stored in glass at -20°C and protected from light (stability = 1 month).

d. Working Standard solutions

Prepare four Working Standard solutions, (WS1–4). These are prepared by diluting aliquots (100, 200, 500, and 1000 µL, respectively) of the Mixed Intermediate Standard solution to a final volume of 10 mL with acetonitrile. The resultant Working Standard solutions thus contain 10.0, 20.0, 50.0, and 100. ng/mL respectively of MG, CV, LMG and LCV. Prepare all Working Standard solutions daily. These solutions may be kept at room temperature but protected from light as much as readily possible (stability = 1 day).

e. Working Internal Standard solution (WIS)

Prepare by taking 400 µL of the Mixed Intermediate Internal standard solution and diluting to a final volume of 10 mL with acetonitrile (final concentration is 40.0 ng/mL). Prepare all Working Standard solutions daily. These solutions may be kept at room temperature but protected from light as much as readily possible (stability = 1 day).

**E. SAMPLE PREPARATION**

Samples collected fresh must be kept cold before and during shipping to the laboratory. Once received at the laboratory, samples must be kept cold or frozen prior to grinding if they cannot be prepared on the day of receipt. Once frozen, the sample should be allowed to thaw, while keeping it as cold as possible. Grind tissue in blender or vertical cutter-mixer until homogeneous. Store samples frozen (< -10 °C) prior to analysis.

**F. ANALYTICAL PROCEDURE**

1. Preparation of Controls and samples

- a. Weigh 2.00 ± 0.02 g of each homogenized sample into 50 ml polypropylene centrifuge tubes.
- b. Weigh 2.00 ± 0.02 g of known blank tissue into 50 mL polypropylene centrifuge tubes. Prepare one for each blank (negative control), recovery (positive control), and check sample that is necessary.
  - i. Fortify recovery (positive control) with 100 µl of WS 2 (1ppb).
- c. Preparation of extracted matrix calibration curve
  - i. Accurately weigh 2.00 ± 0.02 g homogenized negative control tissue into each of four 50 mL disposable centrifuge tubes.

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- ii. Fortify these samples (extracted calibrants 1–4) with 100  $\mu\text{L}$  aliquots of WS1, WS2, WS3, and WS4 respectively. The extracted matrix calibrant samples are fortified with 0.50, 1.0, 2.5, and 5.0 ng/g of all four analytes and 2.0 ng/g of all four internal standards.

2. Extraction Procedure

- a. Fortify each sample, control, and extracted matrix calibration curve tube with 100  $\mu\text{L}$  of Working Internal Standard solution (2.0 ng/g).
- b. Allow samples to equilibrate for 15 min while protected from light.
- c. Add 500  $\mu\text{L}$  of 9.5 g/L hydroxylamine solution to each sample, vortex mix briefly, and allow samples to stand in the dark for  $10 \pm 1$  min.
- d. Add 8 mL of acetonitrile and  $\sim 1$  g anhydrous magnesium sulfate to each sample. Vortex mix tubes for approximately 1 min.
- e. Shake tubes (10 min) using a multitube vortexer.
- f. Centrifuge the tubes ( $2000 \times g$ , 5 min,  $4^\circ\text{C}$ )
- g. Transfer each supernatant to a clean tube for evaporation. Evaporate the supernatant to dryness at  $50 \pm 5^\circ\text{C}$
- h. Reconstitute the extracted matrix calibrant samples and test samples with 800  $\mu\text{L}$  Reconstitution Solution.
- i. Vortex mix all samples sufficiently to break up dried extracts; for example, vortex mixing on high speed for 30 s followed by 10 min of mixing on a multitube vortexer ensures complete dissolution of analytes and internal standards.
- j. Transfer extracts to microcentrifuge tubes, centrifuge at  $20\,000 \times g$  for  $\geq 5$  min, and filter (PVDF, 0.45  $\mu\text{m}$ ) into auto sampler vials for LC-MS/MS analysis.

3. Instrumental Settings

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

LC-MS/MS Analysis:

Instrument operating parameters

- a. Flow rate: 250  $\mu\text{L}/\text{min}$
- b. Injection volume: 20  $\mu\text{L}$
- c. Run time: 10 min.
- d. Column oven:  $30^\circ\text{C}$

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Table 1 - LC Gradient:

Time, min	A, % (ammonium formate buffer)	B, % (acetonitrile)
0	60	40
1	10	90
5	10	90
6	60	40
10	60	40

Table 2 - MS/MS parameters for the Waters Acquity: ESI positive mode.

	SRM, m/z	Collision Energy, eV	Cone Voltage, V	Retention time, min
MG	<b>329 → 313</b>	36	66	2.05
	329 → 208	44	66	2.05
MG-D <sub>5</sub>	334 → 318	34	64	2.04
CV	<b>372 → 356</b>	40	70	2.46
	372 → 340	54	70	2.46
CV-D <sub>6</sub>	378 → 362	40	68	2.44
LMG	<b>331 → 239</b>	32	42	3.50
	331 → 316	20	42	3.50
LMG-D <sub>5</sub>	336 → 239	32	44	3.47
LCV	<b>374 → 358</b>	30	50	3.53
	374 → 239	28	50	3.53
LCV-D <sub>6</sub>	380 → 364	30	50	3.50

Note: The ion used for quantitation is listed in Table 2 in bold.

4. Injection Sequence / Sample Set
  - a. Extracted matrix calibration curve (0.5, 1.0, 2.5, 5.0 ppb)
  - b. Tissue blank
  - c. Recovery (Positive control)
  - d. Intra-laboratory check sample (if needed)
  - e. Samples (Up to 18)
  - f. Reinjection of standard/positive control (for system suitability, if applicable)

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## G. CALCULATIONS

Quantitation is performed by measuring peak areas. Analyte peak areas are divided by corresponding isotopically labeled internal standard peak areas.

$$\text{Peak Area Ratio} = \frac{\text{Analyte Peak Area}}{\text{Isotopically Labeled Internal Standard Peak Area}}$$

A linear regression line is constructed using peak area ratios and standard concentrations. Peak area ratios are plotted as y-axis values and corresponding standard concentrations are plotted as x-axis values. The resulting calibration curve has linear characteristics and the equation  $y = mx + b$  is used to determine the relationship between Peak Area ratios and concentration.

$y = mx + b$ , where...

y = Peak Area Ratio

x = concentration (in ppb)

m = slope

b = y intercept

A similar regression line is generated for each analyte of interest using the same relationship. Expected correlation coefficients are  $r^2 \geq 0.95$ .

Calculate sample concentrations for incurred tissue samples with positive instrument responses using the corresponding regression line.

Peak Heights may be substituted for peak areas if chromatographic peaks display sufficient symmetry.

Note: Should a sample prove to have a concentration that is greater than the highest standard in the calibration curve, the sample should be re-analyzed such that the sample concentration remains bracketed by the calibration curve. The final sample extract may be diluted or the sample re-extracted with a lower sample weight. Sample weight may be adjusted to as little as 1.00 g.

## H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment — Safety glasses, appropriate gloves and lab coat.

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2. Hazards:

<i>Reagent</i>	<i>Hazard</i>	<i>Recommended Safety Procedures</i>
Malachite green	Harmful if ingested. Risk of serious damage to eye. Potential teratogen.	Work in a fume hood.
LeucoMalachite green	Harmful if inhaled or ingested. Causes eye, skin, and respiratory irritation.	Work in a fume hood.
Crystal violet	Harmful if inhaled or ingested.	Work in a fume hood.
LeucoCrystal violet	Harmful if inhaled or ingested. Causes eye, skin, and respiratory irritation.	Work in a fume hood.
Acetonitrile	Flammable. Harmful if inhaled or ingested. Causes eye, skin, and respiratory irritation.	Work in fume hood. Keep away from flame or heat
Methanol	Flammable. Vapors are corrosive to the skin, eyes, and respiratory system. Teratogen.	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

Table 3 – Acceptability criteria

Analyte	Analytical Range	Acceptable Recovery (%)
Malchite Green	≥ 1 ppb	79-119
Leucomalachite Green	≥1 ppb	89-112
Crystal Violet	≥ 1 ppb	90-110
Leucocrystal Violet	≥ 1 ppb	90-111

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2. Acceptability criteria

a. Acceptability criteria for Quantitation:

- i. Retention time for the recoveries and the samples must match the retention time of the 1 ppb calibration curve standard within  $\pm 5\%$ .
- ii. Signal-to-noise ratio for each analyte quantitation peak must be  $\geq 3$ .
- iii. Coefficient of determination ( $r^2$ ) for each calibration curve shall be  $\geq 0.95$ .
- iv. Quantitation ion for all analytes of interest must be present in the positive control. (See table MS/MS Parameters for characteristic ions above.)
- v. All recoveries must meet the acceptable recovery performance standard. (See Table 3)
- vi. Internal standard area for each analyte must be equal to or greater than 25% of the average internal standard area of the calibration curve.
- vii. The peak area ratio for the negative control (blank) must be less than 10% of the peak area ratio of the positive control for each analyte.

b. Acceptability criteria for Confirmation:

- i. Retention time for the recoveries and the samples must match the retention time of the 1 ppb calibration standard within  $\pm 5\%$ .
- ii. Signal-to-noise ratio for all ions must be  $\geq 3$ .
- iii. For the positive control, both characteristic ions for the analytes of interest must be present and the ion ratio of the Qualification Ion to Quantification Ion for each analyte must be within 10% absolute of the 1 ppb calibration standard.
- iv. For a sample to confirm as positive
  - (a) The analyte must quantitate at or above the MLA.
  - (b) The ion ratio of the Qualification Ion to Quantification Ion for each analyte must be within 10% absolute of the 1 ppb calibration standard.

Note: All positive and negative control acceptability requirements apply only to analytes that will be quantitated or confirmed in the sample set.

3. Critical Control Points and Specifications

Record

Acceptable Control

Sample weight

2.00  $\pm$  0.02 g

Evaporation Temperature

50  $\pm$  5°C

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4. 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. 2.  
If unacceptable values are obtained, then:
    - i. Investigate following established procedures.
    - ii. Take corrective action as warranted.
  - c. Sample Condition upon Receipt: cold, unspoiled, and sealed from air.

**J. APPENDIX**

1. References :
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  - b. *Off. J. Eur. Commun.* **L221**, 8(2002)
  - c. *Fed. Regist.* **68**, 25617(2003) <http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/ucm052658.pdf>
  - d. *J. AOAC Int.* **98**, 658(2015) DOI: 10.5740/jaoacint.14-263
  - e. AOAC SMPR 2009.001

**K. APPROVALS AND AUTHORITIES**

1. Approvals on file.
2. Issuing Authority: Director, Laboratory Quality Assurance Staff