Promulgated, Apr 27, 2023
Implemented, Jul 01, 2023
MOHWV0053.00

Method of Test for Veterinary Drug Residues in Foods- Test of Malachite Green, Crystal Violet and their Metabolites

1. Scope

This method is applicable to the determination of malachite green, crystal violet and their metabolites, leucomalachite green and leucocrystal violet, in aquatic products.

2. Method

After extraction and purification, analytes are determined by liquid chromatography/tandem mass spectrometry (LC-MS/MS)

2.1. Equipment

2.1.1. Liquid chromatograph/tandem mass spectrometer.

2.1.1.1. Ion source: electrospray ionization (ESI).

2.1.1.2. Column: Kinetex® C8, 2.6 μm, 2.1 mm i.d. × 50 mm, or an equivalent product.

2.1.2. Homogenizer.

2.1.3. Centrifuge: centrifugal force > 5000 ×g.

2.1.4. Shaker.

2.1.5. Solid phase extraction vacuum manifolds.

2.1.6. Vortex mixer.

2.1.7. pH meter.

2.2. Chemicals

Acetonitrile, HPLC grade;
Methanol, HPLC grade;
n-Hexane, HPLC grade;
Ethyl acetate, HPLC grade;
N,*N*,*N'*,*N'*-tetramethyl-1, 4-phenylenediamine dihydrochloride (TMPD), reagent grade;
Formic acid, reagent grade;
Citric acid, reagent grade;
Disodium hydrogen phosphate, reagent grade;
Ammonium acetate, reagent grade;
Ammonium hydroxide (25%), reagent grade;
Acetic acid, reagent grade;
Hydrogen chloride, reagent grade.

Malachite green oxalate salt (MG, oxalate salt), leucomalachite green (LMG), crystal violet (CV) and leucocrystal violet (LCV), reference standards;

Malachite green-d_5 picrate (MG-d_5 picrate), leucomalachite green-d_5 (LMG-d_5), crystal violet (CV-d_6) and leucocrystal violet-d_6 (LCV-d_6), isotope-labelled internal standards.

2.3. Apparatus and materials

2.3.1. Centrifuge tube: 50 mL, PP.

2.3.2. Volumetric flask: 10 mL, amber.

2.3.3. Cation exchange solid phase extraction cartridge: Oasis MCX, 60 mg, 3 mL, or an equivalent product.

2.3.4. Membrane filter: 0.22 μm, Nylon.

2.4. Reagents

2.4.1. 50% acetonitrile

Dilute 250 mL of acetonitrile with deionized water to 500 mL.

2.4.2. McIlvaines buffer

Dissolve and dilute 9.36 g of citric acid and 1.55 g of disodium hydrogen phosphate with deionized water to 500 mL.

2.4.3. McIlvaines buffer: acetonitrile (1:1, v/v)

Mix McIlvaines buffer and acetonitrile at the ratio of 1:1 (v/v).

2.4.4. TMPD solution

Dissolve and dilute 50 mg of TMPD with methanol to 50 mL.

2.4.5. 0.1 N hydrochloric acid

Add 4.2 mL of hydrochloric acid slowly to 400 mL of deionized water, and dilute with deionized water to 500 mL.

2.4.6. 50% Methanol

Dilute 250 mL of methanol with deionized water to 500 mL.

2.4.7. Elution solution

Mix 5 mL of ammonium hydroxide, 50 mL of ethyl acetate and 45 mL of methanol thoroughly. Prepare freshly before use.

2.4.8. 0.1 M ammonium acetate

Dissolve and dilute 1.93 g of ammonium acetate with deionized water to 250 mL.

2.5. Mobile phase

2.5.1. Solvent A

Mix 50 mL of 0.1 M ammonium acetate and 900 mL of deionized water, adjust pH to 4.5 ± 0.1 with acetic acid, and dilute with deionized water to 1000 mL. Filter with a membrane filter.

2.5.2. Solvent B

Mix 50 mL of 0.1 M ammonium acetate, 900 mL of acetonitrile and 1 mL of formic acid, and dilute with acetonitrile to 1000 mL. Filter with a membrane filter.

2.6. Internal standard solution preparation

Accurately weigh equivalent 10 mg of MG-d_5, LMG-d_5, CV-D_6 and LCV-d_6 isotope-labelled internal standards to each 10-mL volumetric flask, dissolve and dilute with acetonitrile to volume as the internal standard stock solutions, and stored under freezing in the dark. When to use, mix appropriate volume of each internal standard stock solution, and dilute with acetonitrile to 100 ng/mL as the internal standard solution.

2.7. Standard solution preparation

Accurately weigh equivalent 10 mg of MG, LMG, CV and LCV reference standards to each 10-mL volumetric flask, dissolve and dilute with acetonitrile to volume as the standard stock solutions, and stored under freezing in the dark. When to use, mix appropriate volume of each standard stock solution, and dilute with 50% acetonitrile to 100 ng/mL as the standard solution.

2.8. Sample solution preparation

2.8.1. Extraction

Transfer about 1 g of the fine-cut and homogenized sample accurately weighed into a centrifuge tube, and add 10 μL of the internal standard solution, 50 μL of TMPD solution and 10 mL of McIlvaines buffer: acetonitrile (1:1, v/v). Vortex-mix, centrifuge at 5000 ×g for 3 min, and collect the supernatant. Add 5 mL of McIlvaines buffer: acetonitrile (1:1, v/v) to the residue, and repeat the extraction step once. Combine the supernatants for purification.

2.8.2. Purification

Transfer the solution for purification from section 2.8.1 into an Oasis MCX cartridge prerinsed with 2 mL of methanol, 2 mL of deionized water and 2 mL of McIlvaines buffer solution in order, and discard the eluent. Wash the cartridge with 2 mL of 0.1 N HCl and 5 mL of deionized water, and discard the eluents. Dry the cartridge by vacuum suction. Wash the cartridge with 3 mL of 50% methanol and 3 mL of *n*-hexane, and discard the eluents. Dry the cartridge by vacuum suction, add 5 mL of the elution solution, and collect the eluent. Evaporate the eluent to near dryness by gently flushing with a stream of nitrogen at 50°C. Add 1 mL of 50% acetonitrile to the residue, vortex-mix to dissolve, and filter with a membrane filter. Take the filtrate as the sample solution.

2.9. Calibration standard curve

Take a blank sample, add 2-50 μL of the standard solutions and 10 μL of the internal standard solution, and follow the procedure described in section 2.8 to obtain the calibration standard solutions. Operate LC-MS/MS according to the following conditions. Establish the calibration standard curve of MG, LMG, CV or LCV by the ratios of peak area of MG, LMG, CV or LCV to that of the internal standard vs. the added concentrations in the range of 0.2-5 ng/mL.

LC-MS/MS operating conditions(note):

Column: Kinetex® C8, 2.6 μm, 2.1 mm i.d. × 50 mm

Column temperature: 35°C.

Mobile phase: a gradient program of solvent A and solvent B is as follows.

Time (min)	A (%)	B (%)
0.0 → 8.0	95 → 0	5 → 100
8.0 → 13.0	0 → 0	100 → 100
13.0 → 13.5	0 → 95	100 → 5
13.5 → 15.5	95 → 95	5 → 5

Flow rate: 0.2 mL/min.

Inject volumn: 10 μL.

Capillary voltage: 2.5 kV.

Ionization mode: ESI^+.

Ion source temperature: 100°C.

Desolvation temperature: 400°C.

Cone gas flow rate: 50 L/hr.

Desolvation flow rate: 850 L/hr.

Detection mode: multiple reaction monitoring mode (MRM). Detection ion pair, cone voltage and collision energy are shown as follows:

Analyte	Ion pair: Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
MG	329 > 313*	10	34
	329 > 208	10	32
LMG	331 > 239*	34	29
	331 > 316	34	20
CV	372 > 356*	54	36
	372 > 340	54	52
	372 > 235	54	56
LCV	374 > 358*	78	29
	374 > 239	78	30
	374 > 253	78	30
MG-d_5 (I.S.)	334 > 318	12	35
LMG-d_5 (I.S.)	336 > 239	12	29
CV-d_6 (I.S.)	378 > 362	14	40
LCV-d_6 (I.S.)	380 > 364	12	30

*The quantitation ion. The qualitative ion pair should be selected at least one ion pair depending on the matrix.

Note: All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

2.10. Identification and quantification:

Accurately inject 10 μL of the sample solution and the calibration standard solutions into LC-MS/MS separately, and operate according to the condition described in section 2.9. Identify each antibiotic dye or their metabolites based on the retention time and the relative ion intensities(note1). Calculate the amount of each

antibiotic dye in the sample by the following formula(note2):

$$\text{The amount of each antibiotic dye in the sample (ppb)} = \frac{\sum C \times V}{M}$$

Where,

C: the concentration of each antibiotic dye or its metabolite in the sample solution calculated by the calibration standard curve (ng/mL)

V: the final make-up volume of the sample (1 mL)

M: the weight of the sample (g)

Note: 1. Relative ion intensities are calculated by peak areas of qualitative ions divided by peak areas of quantitation ions. Maximum permitted tolerances of relative ion intensities by LC-MS/MS are as follows:

Relative ion intensity (%)	Tolerance (%)
> 50	± 20
> 20～50	± 25
> 10～20	± 30
≤ 10	± 50

2. The amount of each antibiotic dye in the sample for MG is calculated by the sum of MG and LMG, and that for CV is calculated by the sum of CV and LCV.

Remark

1. The limits of quantitation (LOQs) for malachite green (the sum of malachite green and leucomalachite green) and crystal violet (the sum of crystal violet and leucocrystal violet) are all 0.5 ppb.
2. Further validation shall be performed when interfering compounds appear in samples.
3. Due to the structural properties of crystal violet (CV), it can be easily adsorbed to balances, laboratory benches, laboratory coats, gloves, pens, glassware, etc., electrostatically and generates the background signal. Therefore, when performing sample preparation, it should be ensured that the working environment is not contaminated to prevent the background value of CV from affecting the test results.

Reference

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