Analytical Method for Malachite Green
(Target to Animal and Fishery Products)

The target compounds to be determined are malachite green and leucomalachite green.

1. Instrument

Liquid chromatograph-tandem mass spectrometer (LC-MS/MS)

2. Reagents

Use the reagents listed in Section C ***Reagents/Test Solutions, Etc.***, Part II ***Food Additives***, except the following. Reagents designated as "special grade" in this section must meet the requirements for "special grade" specified in the Japan Industrial Standards for the reagents.

Acetonitrile: Use a reagent not containing any substance that may interfere with the analysis of the target compounds.

Acetone: Use a reagent not containing any substance that may interfere with the analysis of the target compounds.

Ethanol: Use a reagent not containing any substance that may interfere with the analysis of the target compounds.

Ammonium formate: Ammonium formate (special grade).

Citric acid (anhydrous): Citric acid (anhydrous) (special grade).

Sulfonate-modified divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge (500 mg): A polyethylene tube of 12-13 mm in inside diameter packed with 500 mg of sulfonate-modified divinylbenzene-*N*-vinylpyrrolidone copolymer, or a cartridge equivalent to the specified one in separation capability.

Quaternary ammonium salt-modified divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge (150 mg): A polyethylene tube of 12-13 mm in inside diameter packed with 150 mg of quaternary ammonium salt-modified divinylbenzene-*N*-vinylpyrrolidone copolymer, or a cartridge equivalent to the specified one in separation capability.

50 mmol/L ammonium formate buffer (pH 3.5): Dissolve 3.15 g of ammonium formate in 990 mL of water. Adjust pH to 3.5 with formic acid, and add water to make exactly 1,000 mL.

3. Reference standard

Reference standard of malachite green oxalate: Contains not less than 98% of malachite green oxalate.

Reference standard of leucomalachite green: Contains not less than 98% of leucomalachite green.

4. Procedure

a. Extraction

Weigh sample accurately and add half amount in weight ratio of 15 w/w% of dibutylhydroxytoluene-ethanol solution and half amount in weight ratio of 50 w/w% of citric acid solution, respectively. Homogenize and take the sample equivalent to 10.0 g (5.00 g for fat). Add 100 mL of acetone, homogenize, and filter with suction using glass fiber filter. Add 50 mL of acetone (10 mL of water and 50 mL of acetone for honey) to the residue on the filter, homogenize, and filter as described above. Combine the resulting filtrates, and add acetone to make exactly 200 mL. Take exactry a 1 mL (2 mL for fat) aliquot of the solution and add 4 mL of 2 vol% formic acid.

b. Clean-up

Add 5 mL each of acetonitrile and 2 vol% formic acid to a sulfonate-modified divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge (500 mg) sequentially and discard the effluents. Add 5 mL of acetonitrile/ammonia water (9:1, v/v) to a quaternary ammonium salt-modified divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge (150 mg) and discard the effluent. Transfer the solution obtained in "a. Extraction" to the sulfonate-modified divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge, add 5 mL of acetonitrile, and discard the effluent. Connect the quaternary ammonium salt-modified divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge to the bottom of the sulfonate-modified divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge, elute with 10 mL of acetonitrile/ammonia water (9:1, v/v), and take the eluate. Add acetonitrile/ammonia water (9:1, v/v) to the eluate to make exactly 10 mL, and use this solution as the test solution.

5. Measurement

a. Calibration curve

Dissolve reference standards of malachite green oxalate and leucomalachite green in acetone to make 500 mg/L respectively (for malachite green oxalate, adjust the concentration as malachite green), and use these solutions as stock standard solutions. Mix each stock standard solution appropriately, dilute with acetonitrile/ammonia water (9:1, v/v), and prepare standard solutions of several concentrations. Inject each standard solution to LC-MS/MS, and make calibration curves by peak-height or peak-area method. When the test solution is prepared following the above procedure, the sample containing 0.002 mg/kg of

malachite green and leucomalachite green gives the test solution of 0.00001 mg/L in concentration.

b. Quantification

Inject the test solution in LC-MS/MS, and calculate the concentration of malachite green and leucomalachite green from the calibration curve made in "a. Calibration curve".

c. Confirmation

Confirm using LC-MS/MS.

d. Measurement conditions

(Example)

Column: Octadecylsilanized silica gel, 2.1 mm in inside diameter, 150 mm in length and 5 μm in particle diameter

Column temperature: 40°C

Mobile phase: Linear gradient from acetonitrile/50 mmol/L ammonium formate buffer (pH 3.5) (3:7, v/v) to (9:1, v/v) in 15 min and hold for 10 min.

Ionization mode: ESI (+)

Major monitoring ions (*m/z*):

Malachite green: Precursor ion 329, product ions 313, 165

Leucomalachite green: Precursor ion 331, product ions 316, 239

Injection volume: 10 μL

Expected retention time

Malachite green: 8 min

Leucomalachite green: 16 min

6. Limit of Quantification

Malachite green: 0.002 mg/kg

Leucomalachite green: 0.002 mg/kg