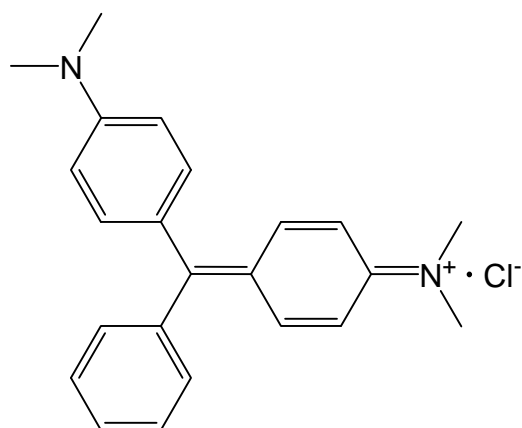


16 Malachite green 17 Leucomalachite green

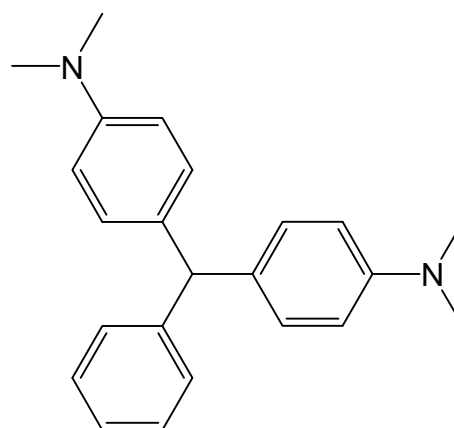
Malachite green (hydrochloride)



N-[4-[[4-(dimethylamino)-phenyl]
phenylmethylene]-2,5-cyclohexadien-1-ylidene
]-*N*-methylmethanaminium chloride

C₂₃H₂₅ClN₂ MW: 364.9
CAS No.: 569-64-2

Leucomalachite green



4,4'-benzylidenebis(*N,N*-dimethylaniline)

C₂₃H₂₆N₂ MW: 330.5
CAS No.: 129-73-7

【Outline of malachite green and leucomalachite green】

Malachite green is a green synthetic pigment used as triphenylmethane dye to industrially color fibers and the like. It has antibacterial activity, having been used as an agent for treatment of *saprolegniasis* of fish. However, its carcinogenicity is suggested and genetic toxicity is suspected, and therefore, this agent is used only for aquarium fish.

Leucomalachite green is a metabolite generated by reduction of malachite green in the living body.

《The standard value in Food Sanitation Act》

Not to be detected (lower detection limit: 0.002 ppm)

【Methods listed in the Feed Analysis Standards】

1. The simultaneous analysis method (No. 1. Fish meal and formula feed) of malachite green and leucomalachite green with a liquid chromatograph tandem-type mass spectrometer

[Feed Analysis Standards Chapter 8, Section 1, 16.1 and 17.1]

**Target chemicals: Malachite green and leucomalachite green
(2 components)**

Scope of application: Fish meal and formula feed

A. Reagent preparation

- 1) Malachite green and leucomalachite green standard stock solutions ^{Note 2}: Place 10 mg of malachite green [C₂₃H₂₅ClN₂] ^[1] and 10 mg of leucomalachite green [C₂₃H₂₆N₂] ^[1] exactly measured in respective 100 mL volumetric flasks, add methanol for dissolving, and further add the solvent up to the gauge line to prepare the malachite green and leucomalachite green standard stock solutions (1 mL of these solutions contain amounts of malachite green and leucomalachite green equivalent to 100 µg, respectively).
- 2) Stable isotope element-labeled substance standard stock solutions and mixed internal standard solutions: Place 5 mg of stable isotope element-labeled malachite green ^{Note 3} (MG-d₅) ^[1] and 5 mg of stable isotope element-labeled leucomalachite green ^{Note 3} (LMG-d₆) ^[1] exactly measured in 50 mL volumetric flasks, respectively, add methanol for dissolving, and further add the solvent up to the gauge line to prepare respective stable isotope element-labeled substances standard stock solutions (1 mL of these solutions contain amounts of MG-d₅ and LMG-d₆ equivalent to 100 µg, respectively).
Then, mix a definite amounts of respective standard stock solutions, dilute exactly with acetonitrile-water (1:1) to prepare the mixed internal standard solution containing amounts of MG-d₅ and LMG-d₆ equivalent to 25 ng/mL, respectively.
- 3) Standard solution for preparing the calibration curve: At the time of use, dilute definite amounts of the malachite green and leucomalachite green standard stock solutions as well as a definite amount of the mixed internal standard solution exactly with acetonitrile-water (1:1) to prepare several standard solutions containing 0.5-200 ng/mL of malachite green and 0.5-200 ng/mL of leucomalachite green as well as 5 ng of MG-d₅ and 5 ng of LMG-d₆, for preparing the calibration curve.
- 4) Citric acid-phosphate buffer solution: Dissolve 63.0 g of citric acid monohydrate with water to make a total volume of 1,000 mL, and add approximately 300 mL of disodium hydrogenphosphate solution (0.6 mol/L) to adjust the pH at 3.0.

B. Quantification

Extraction: Place 5 g of the analysis sample exactly measured in a stoppered 100 mL centrifuging tube, add 10 mL of citric acid-phosphate buffer solution and 30 mL of acetonitrile, and allow still standing for 5 min. Then, add 1 mL of the stable isotope element-labeled mixed internal standard solution to this centrifuging tube, and stir with an ultrahigh-speed crusher ^[2] for 1 min for extraction.

Wash the ultrahigh-speed crusher with a small amount of acetonitrile, add the washings to the extracted solution, and centrifuge at 1,300×g for 5 min to obtain the supernatant as a sample solution for purification.

Purification: Place the sample solution in a 100 mL separating funnel containing 25 mL of dichloromethane and 5 g of sodium chloride, rinse the centrifuging tube previously containing the sample solution with a small amount of acetonitrile, and add the washings to the sample solution. Shake the separating funnel for 1 min, allow still standing, and filter the

acetonitrile-dichloromethane layer (upper layer) through a paper filter (No. 5A) loaded in layers with an appropriate amount (20 g or more) of sodium sulphate (anhydrous) ^{Note 4} placed on an infundibulum into a 200 mL recovery flask ^[3]. Wash that separating funnel and the paper filter sequentially with a small amount of dichloromethane, filter the washings in a similar way, and mix the filtered solutions.

Concentrate the filtered solution in a water bath under a reduced pressure at 40 °C or lower to approximately 1 mL, dry to solid with nitrogen gas, and dissolve the residue by adding 5 mL of acetonitrile-acetic acid (19:1) to obtain a sample solution for column treatment.

Column treatment: Wash a benzene-sulfonylpropylsilylated silica gel minicolumn (500 mg) ^{Note 5} with 5 mL of acetonitrile-acetic acid (19:1). Place the sample solution in the minicolumn, and naturally effuse until the fluid level reaches the top of the packing material. Wash the recovery flask previously containing the sample solution with a small amount of acetonitrile-acetic acid (19:1), and add the washings to the minicolumn to effuse in a similar way. Then, add 2.5 mL of acetone, 5 mL of methanol, and 5 mL of acetonitrile sequentially to the minicolumn to effuse in a similar way.

Dry the minicolumn by aeration for 5 min ^{Note 6}, place a 50 mL recovery flask under the minicolumn, and add 10 mL of acetonitrile-ammonia water (19:1) to the minicolumn to elute malachite green and leucomalachite green.

Concentrate the eluent under a reduced pressure in a water bath at 40 °C or lower almost to dryness, and send nitrogen gas to obtain the dry matter. Add exactly 5 mL of acetonitrile-water (1:1) to dissolve the residue, and filter through a membrane filter (pore diameter: 0.45 µm or less) to obtain a sample solution for determination with a liquid chromatograph tandem-type mass spectrometer.

Measurement with a liquid chromatograph tandem-type mass spectrometer: Inject respective 10 µL of sample solution and each mixed standard solution to a liquid chromatograph tandem-type mass spectrometer to obtain the selective reaction monitoring chromatogram.

Measurement conditions (example)

(Part of liquid chromatograph)

Column: Octadecylsilylated silica-gel column (internal diameter: 2.1 mm, length: 150 mm, particle diameter: 5 µm) ^{Note 7}

Eluent: 0.3 v/v% ammonia water• 2 v/v% formic acid solution-water-acetonitrile ^{Note 8}
(10:81:9)-0.3 v/v% ammonia water• 2 v/v% formic acid solution-water-acetonitrile
(10:9:81) (7:3) (1 min retention)→7 min→0.3 v/v% ammonia water• 2 v/v% formic acid solution-water-acetonitrile (10:9:81) (10 min retention)

Flow rate: 0.25 mL/min

Column temperature: 40 °C

(Part of tandem-type mass spectrometer, Example 1 ^{Note 9})

Ionization method: electrospray ionization (ESI) method (positive ion mode)

Nebulizer gas pressure: 340 kPa

Dry gas temperature: 350 °C

Capillary voltage: 4 kV

Fragmenter voltage: See the table given below

Collision energy: See the table given below

Monitor ion: See the table given below

Table. Monitor ion conditions for each substance

Name of substance	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	Confirmation ion (<i>m/z</i>)	Fragmenter voltage (V)	Collision energy (V)
Malachite green	329	313	208	100	40
Malachite green d ₅	334	318	-	100	40
Leucomalachite green	331	239	316	100	25
Leucomalachite green d ₆	337	240	-	100	25

(Part of tandem-type mass spectrometer, Example 2^{Note 10})

Ionization method: Electrospray ionization (ESI) method (positive ion mode)

Ion source temperature: 150 °C

Desolvation temperature: 400 °C

Capillary voltage: 3.5 kV

Cone voltage: See the table given below.

Collision energy: See the table given below.

Monitor ion: See the table given below.

Table. Monitor ion conditions for each substance

Name of substance	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	Confirmation ion (<i>m/z</i>)	Fragmenter voltage (V)	Collision energy (V)
Malachite green	329	313	208	100	40
Malachite green d ₅	334	318	-	100	40
Leucomalachite green	331	239	316	100	25
Leucomalachite green d ₆	337	240	-	100	25

Calculation: Obtain the peak areas of malachite green, leucomalachite green, MG-d₅ and LMG-d₆ from the selective reaction monitoring chromatogram^[4], and prepare the calibration curve by the internal standard method to calculate the amounts of malachite green and leucomalachite green in the sample.

Note 1. Since displacement of the unchanged body and metabolite of malachite green possibly occurs, operate the experiment promptly.

2. 10.0 mg of malachite green [C₂₃H₂₅ClN₂] is equivalent to 12.7 mg of malachite green oxalate [C₅₂H₅₄N₄O₁₂] .

3. Malachite green and leucomalachite green used as a stable isotope element-label are malachite green d₅ and leucomalachite green d₆ or equivalent ones.

4 A reagent for PCB test or equivalent one.

5 Bond Elut SCX (Varian) with an appropriate reservoir or an equivalent one.

- 6 Dry with aeration using a suction manifold.
- 7 ZORBAX Eclipse XDB-C18 (Agilent Technologies, the retention times of malachide green and leucomalachide green in this measurement condition are approximately 8 min and 14 min, respectively) or an equivalent one.
- 8 A reagent for LC/MS (Kanto Chemical or Wako Pure Chemical) or an equivalent one.
- 9 An example condition for Agilent 6410 Triple Quad LC/MS (Agilent Technologies)
- 10 An example condition for Micromass Quattro micro API (Waters)

《Summary of analysis method》

This method is intended to determine the amount of malachide green and leucomalachide green in a fish meal and formula feed, by extracting, purifying with a cation exchange minicolumn, and quantifying (internal standard method) using a liquid chromatograph tandem-type mass spectrometer.

The flow sheet of analysis method is shown in Fig. 8.2.2-1.

- Sample: 5 g
- Place in stoppered 100 mL centrifuging tube.
 - 10 mL of citric acid-phosphoric acid buffer solution and 30 mL of acetonitrile
 - Still standing for 5 min
 - Add 1 mL of internal standard solution (25 ng/mL).
- Homogenize (for 1 min)
- Wash the shaft with a small amount of acetonitrile.
 - Centrifuge (at 1,300×g for 5 min).
- Supernatant
- Transfer to a 100 mL separating funnel containing 25 mL of dichloromethane and 5 g of sodium chloride.
 - Wash the centrifuging tube with a small amount of acetonitrile.
 - Shake for 1 min.
 - After still standing, remove the lower layer (water layer containing sodium chloride).
- Upper layer (acetonitrile-dichloromethan layer)
- After dehydration with an appropriate amount of sodium sulfate (unhydrus), place in a 200 mL recovery flask.
 - Wash the separating funnel and paper filter with a small amount of dichloromethane.
 - Concentrate under reduced pressure (at 40 °C or lower, to approximately 1 mL) and dry to solid with nitrogen gas.
 - Acetonitrile-acetic acid (19:1): 5 mL
- Bond Elut SCX cartridge (connected with a 10 mL reservoir)
- Wash with 5 mL of acetonitrile-acetic acid(19:1).
 - Load the sample solution
 - Wash with a small amount of acetonitrile-acetic acid (19:1).
 - Flow out sequentially with 2.5 mL of acetone, 5 mL of methanol and 5 mL of acetonitrile.
- } Naturally
flow down
- Dry with aeration (for 5 min)
- Place a 50 mL recovery flask under the minicolumn.
 - Elute (naturally flow down) with 10 mL of acetonitrile-ammonia water (19:1).
 - Concentrate under reduced pressure (40 °C or lower) and dry to solid with nitrogen gas.
 - Acetonitrile-water (1:1): 5 mL
 - Membrane filter (0.45 μm)
- LC-MS/MS

Fig. 8.2.2-1 Simultaneous analysis method of malachite green and leucomalachite green with a liquid chromatograph tandem-type mass spectrometer

Reference: Toshiichi Komoriya: Research Report of Animal Feed, 33, 101 (2008)

History in the Feed Analysis Standards: 【31】 new

《Validation of analysis method》

• Recovery rate and repeat accuracy

Added component	Type of sample	Concentration ($\mu\text{g}/\text{kg}$)	Repeat	Recovery rate (%)	Repeat accuracy RSD (% or less)
Malachite green	Fish meal	5.0-100	3	78.1-81.4	2.4
	Formula feed for red sea bream	100	3	73.7	1.6
	Formula feed for growing chick	5.0-100	3	84.2-87.9	1.8
	Formula feed for piglet	5.0-100	3	85.6-90.5	1.6
Leucomalachite green	Fish meal	5.0-100	3	77.1-85.0	3.7
	Formula feed for red sea bream	100	3	93.9	1.9
	Formura feed for growing chick	5.0-100	3	94.5-95.2	1.9
	Formula feed for piglet	5.0-100	3	98.3-99.0	3.1

• Cooperative testing

Analysis component	Type of sample	No. of labs	Concentration ($\mu\text{g}/\text{kg}$)	Recovery rate (%)	Repeat accuracy in room		HorRat
					RSD _r (%)	Reproducibility RSD _R (%)	
Malachite green	Fish meal	9	2.0	86.1	3.9	7.4	0.34
	Formula feed for piglet	9	2.0	93.8	4.0	5.4	0.25
	Formula feed for fish	3	5.0	101.4	1.7	2.7	0.12
		3	10.0	94.3	11.0	12.3	0.56
Leucomalachite green	fish meal	9	2.0	91.3	4.6	16.4	0.74
	Formula feed for piglet	9	2.0	100.4	3.9	6.1	0.28
	Formula feed for fish	3	5.0	101.7	1.0	7.8	0.35
		3	10.0	101.1	1.7	8.2	0.37

• Lower detection limit: 2 $\mu\text{g}/\text{kg}$ for malachite green and leucomalachite green, respectively

《Notes and precautions》

- [1] Respective standards are available from Hayashi Pure Chemical.
- [2] Use HG-200 (Hsiangtai, generator shaft: $\phi 12 \times 170$ mm) or an equivalent one as the main body to homogenize at approximately 15,000 rpm.
- [3] After removing citric acid-phosphate buffer solution (lower layer) containing sodium chloride from underneath, filter the acetonitrile-dichloromethane layer (upper layer).
- [4] A sample of selective reaction monitoring (SRM) chromatogram in fish meal is shown in Fig. 8.2.2-2.

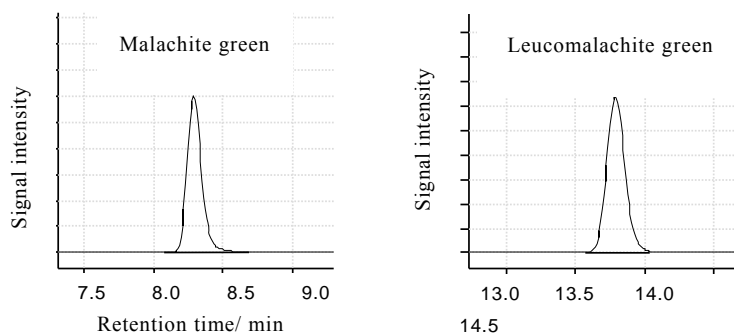


Fig. 8.2.2-2 SRM chromatograms of malachite green and leucomalachite green
(Amounts equivalent to 5 $\mu\text{g}/\text{kg}$ were added, respectively)

Measurement conditions are according to Part of liquid chromatograph (example) and Part of tandem-type mass spectrometer (example 1).

2. The simultaneous analysis method (No. 2. Fish oil) of malachite green and leucomalachite green with a liquid chromatograph tandem-type mass spectrometer

[Feed Analysis Standards Chapter 8, Section 1, 16.2 and 17.2]

**Target chemicals: Malachite green and leucomalachite green
(2 components)**

Scope of application: Fish meal

A. Reagent preparation

- 1) Malachite green and leucomalachite green standard stock solutions ^{Note 2}: Place 10 mg of malachite green [$C_{23}H_{25}ClN_2$] ^[1] and 10 mg of leucomalachite green [$C_{23}H_{26}N_2$] ^[1] exactly measured in 100 mL volumetric flasks, respectively, add methanol for dissolving, and further add the solvent up to the gauge line to prepare the malachite green and leucomalachite green standard stock solutions (1 mL of these solutions contain amounts of malachite green and leucomalachite green equivalent to 100 µg, respectively).
- 2) Stable isotope element-labeled substance standard stock solution and mixed internal standard solution: Place 5 mg of the stable isotope element-labeled malachite green ^{Note 3} (MG-d₅)^[1] and 5 mg of the stable isotope element-labeled leucomalachite green ^{Note 3} (LMG-d₆)^[1] exactly measured in 50 mL volumetric flasks, respectively, add methanol for dissolving, and further add the solvent up to the gauge line to prepare respective stable isotope element-labeled substance standard stock solutions (1 mL of these solutions contain amounts of MG-d₅ and LMG-d₆ equivalent to 100 µg, respectively).
Then, mix definite amounts of respective standard stock solutions, dilute exactly with acetonitrile to prepare the mixed internal standard solutions containing amounts of MG-d₅ and LMG-d₆ equivalent to 1 µg/mL, respectively.
- 3) Standard solution for preparing the calibration curve: At the time of use, dilute definite amounts of the malachite green and leucomalachite green standard stock solutions as well as the mixed internal standard solution exactly with acetonitrile-water (1:1) to prepare several standard solutions containing 0.5-20 ng/mL of malachite green and 0.5-20 ng/mL of leucomalachite green as well as 5 ng of MG-d₅ and 5 ng LMG-d₆ for preparing the calibration curve.

B. Quantification

Extraction: Place 1 g of the analysis sample exactly measured in a stoppered 100 mL centrifuging tube, and add exactly 50 µL of the mixed internal standard solution. Then, add 40 mL of acetonitrile-acetic acid (19:1) and 10 mL of acetonitrile-saturated hexane, and mix while shaking for 10 min for extraction. Centrifuge at 1,300×g for 5 min, discard the hexane layer (upper layer) to

obtain the acetonitrile layer (lower layer) ^[2] as a sample solution.

Column treatment: Wash a benzenesulfonylpropylsilylated silica-gel minicolumn (500 mg) ^{Note 4} with 5 mL of acetonitrile-acetic acid (19:1). Place 4 mL of the sample solution exactly measured in the minicolumn, and effuse until the fluid level reaches the top of the packing material by natural flowing down. Add 2.5 mL of acetone and 5 mL of acetonitrile sequentially to the minicolumn for effusing in a similar way.

Place a 50 mL recovery flask under the minicolumn, and add 10 mL of acetonitrile-ammonia water (19:1) to the minicolumn to elute malachite green and leucomalachite green.

Concentrate the eluate in a water bath at 40 °C or lower under reduced pressure to be almost dryness, send nitrogen gas to be dry matter, add exactly 1 mL of acetonitrile-water (1:1) to dissolve the residue, centrifuge at 5,000×g for 5 min to obtain the supernatant as a sample solution for measurement with a liquid chromatograph tandem-type mass spectrometer.

Measurement with a liquid chromatograph tandem-type mass spectrometer^{[3][4]}: Measure according to 2-(3)-B, Measurement with a liquid chromatograph tandem-type mass spectrometer.

Calculation: Calculate according to 2-(3)-B, Calculation.

Note 1. Since displacement of the unchanged body and metabolite of malachite green possibly occurs, operate the experiment promptly.

2. 10.0 mg of malachite green [C₂₃H₂₅ClN₂] is equivalent to 12.7 mg of malachite green oxalate [C₅₂H₅₄N₄O₁₂] .

3. Malachite green and leucomalachite green to be used for stable isotope element-labels are malachite green d₅ and leucomalachite green d₆ or equivalent ones.

4. Bond Elut SCX (Varian) connected with a reservoir with an appropriate capacity, or an equivalent one.

《Summary of analysis method》

This method is intended to determine the amount of malachite green and leucomalachite green in fish oil, by extracting, purifying with a cation exchanging minicolumn, and quantifying with a liquid chromatograph tandem-type mass spectrometer (internal standard method).

The flow sheet of analysis method is shown in Fig. 8.2.3-1.

- Sample: 1 g
- Place in a stoppered 100 mL centrifuging tube
 - Add 50 μ L of the internal standard solution (1 μ g/mL)
 - 40 mL of acetonitrile-acetic acid (19:1) and 10 mL of acetonitrile-saturated hexane
- Stir for 10 min
- Centrifuge at 1,300 \times g for 5 min
 - Remove the upper layer (hexane layer) with a Komagome pipette, and use the lower layer (acetonitrile layer) as a sample solution.
- Bond Elut SCX cartridge (connected with a 10 mL reservoir)
- Wash with 5 mL of acetonitrile-acetic acid (19:1)
 - Load 4 mL of sample solution
 - Flow out with 2.5 mL of acetone. } Naturally flow down
 - Flow out with 5 mL of acetonitrile }
 - Place a 50 mL recovery flask under the minicolumn
 - Elute with 10 mL of acetonitrile-ammonia (19:1) (naturally flow down)
 - Concentrate under reduced pressure (40 °C or lower) and dry to solid with nitrogen gas
 - 1 mL of acetonitrile-water (1:1)
 - High speed centrifuge at 5,000 \times g for 5 min
- LC-MS/MS

Fig. 8.2.3-1 Simultaneous analysis method of malachite green and leucomalachite green with a liquid chromatograph tandem-type mass spectrometer

Reference: Toshiichi Komori: Research Report of Animal Feed, 34, 43 (2009)

History in the Feed Analysis Standards: 【33】 new

《Validation of analysis method》

• Recovery rate and repeat accuracy

Added component	Type of sample	Concentration (μ g/kg)	Repeat	Recovery rate (%)	Repeat accuracy RSD (% or less)
Malachite green	Purified fish oil 1	2-100	3	93.3-97.4	5.9
	Purified fish oil 2	2-100	3	89.9-98.0	3.3
	Unpurified fish oil 1	2-100	3	91.8-98.1	10
	Unpurified fish oil 2	2-100	3	94.2-101	4.6
Leucomalachite green	Purified fish oil 1	2-100	3	99.5-107	3.0
	Purified fish oil 2	2-100	3	103-112	3.9
	Unpurified fish oil 1	2-100	3	94.6-97.7	1.7
	Unpurified fish oil 2	2-100	3	99.4-105	1.6

• Cooperative testing

Analysis component	Type of sample	No. of labs	Concentration (μ g/kg)	Recovery rate (%)	Repeat accuracy in room		HorRat
					RSD _r (%)	Reproducibility RSD _R (%)	
Malachite green	Purified fish oil	8	5.0	87.4	6.8	13	0.60
	Unpurified fish oil	8	5.0	85.9	2.5	12	0.56
Leucomalachite green	Purified fish oil	8	5.0	108	3.8	8.2	0.37
	Unpurified fish oil	8	5.0	101	4.2	6.2	0.28

- Lower detection limit: 2 μ g/kg for malachite green and leucomalachite green, respectively.

《Notes and precautions》

- [1] Respective standards are available from Hayashi Pure Chemical.
- [2] Some turbidity does not affect the quantification.
- [3] Measuring the sample solution just after the standard solution may cause carry over for malachite green; therefore, note the necessity of washing the program of the syringe.
- [4] A sample of selective reaction monitoring (SRM) chromatogram is shown in Fig. 8.2.3-2.

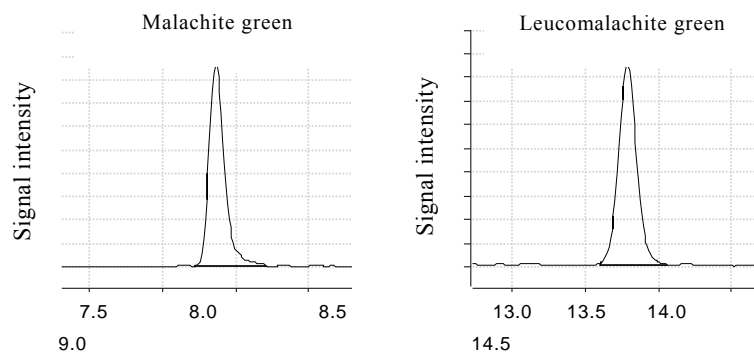


Fig 8.2.3-2 A SRM chromatogram of malachite green and leucomalachite green
(Amounts equivalent to 2 $\mu\text{g}/\text{kg}$ were added, respectively)

Measurement conditions are the same as Fig. 8.2.2-2 in this section 2.