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LC–MS/MS Analysis of Malachite Green and Crystal Violet using Pursuit™ XRs

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Introduction

Malachite green (MG) and crystal violet (CV) are synthetic pigments mainly used for dyeing. In addition, they are used to treat water-borne infectious diseases, particularly in fish and eels, as they have anti-bacterial properties. However, MG is a suspected carcinogen and hence, its use in aquaculture is currently prohibited. The Japanese Ministry of Health, Labor and Welfare has required testing for these compounds and metabolites (MG is enzymatically metabolized in vivo to leuco-malachite green) using a high performance liquid chromatograph mass spectrometer (LC/MS). However, the best option for maximum sensitivity and selectivity is to use triple-quadrupole MS (LC-MS/MS).

Instrumentation

HPLC Instrument: Varian 212 HPLC
Mass Spectrometer: Varian 320 MS/MS
Autosampler: Prostar™ 410

Conditions

MS Ionization Method: ESI
Measurement: MRM (positive/negative automatic change mode)
Collision Gas: Ar (2 mTorr)
Sample Injection Volume: 20 µL
LC Column: Pursuit XRs C18 3µm, 150mm × 2mm
Sample Loop: 20 µL
Column Oven Temperature: 40 °C
Flow Rate: 0.2 mL/min
Mobile Phase:
A: 0.01 % Formic acid aqueous solution
B: 0.01 % Formic acid in acetonitrile

Time (min)	0	0.5	4	12	12	20
Component A %	10	10	95	95	10	10
Component B %	90	90	5	5	90	90

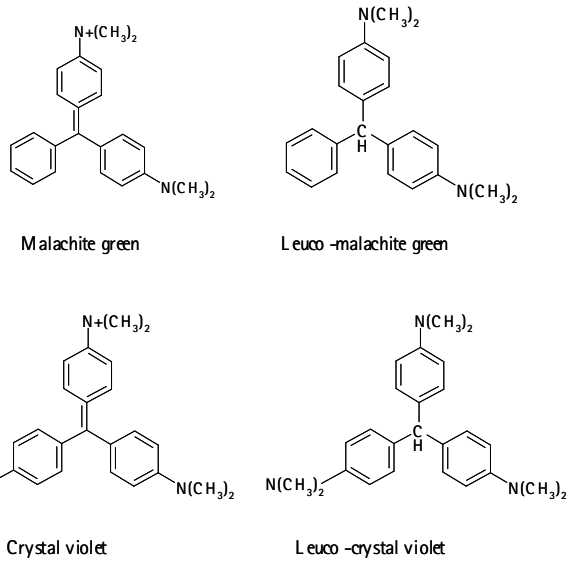

Figure 1. Chemical structures.

Table 1. MS/MS conditions.

	Precursor Ion	Product Ion	CID Energy	Dwell Time
Malachite Green	329	208	30	0.1
Malachite Green	329	313	28	0.1
Leucomalachite Green	331	239	25	0.1
Leucomalachite Green	331	316	15	0.2
Crystal Violet	372	284	45	0.2
Crystal Violet	372	356	30	0.1
Leucocrystal Violet	374	238	20	0.2
Leucocrystal Violet	374	358	25	0.2

Method

This method is suitable for testing malachite green and its metabolite (leucomalachite green) in fish and sea foods.

Technique: Liquid chromatography/tandem mass spectrometry, LC/MS/MS
Ion source: positive ion electrospray ionization, ESI+
Column: Endcapped UPS C8, 3-5 mm, 2.0mm x 50 mm or similar
Homogenizer
Centrifuge: >2400 rpm
Shaker
Solid phase extraction vacuum manifolds
Vortex mixer
pH meter

Chemicals
acetonitrile, methanol, n-hexane, and ethyl acetate are HPLC grade; N,N,N',N'-tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD), formic acid, citric acid, disodium hydrogen phosphate (or sodium phosphate, dibasic), ammonium acetate, 25 % ammonia, acetic acid, and hydrochloric acid are analytical grade; malachite green MG standard, leucomalachite green LMG standard, isotope labeled malachite green standard (MG-d5 picrate), isotope labeled leucomalachite green standard (LMG-d5)

Other Materials
Plastic Centrifugation Tubes: 50 mL
Cation Exchange Solid Phase Extraction Cartridge: Bond Elut Plexa™ PCX, 60 mg, 3 mL or similar
Filter: pore size 0.22 and 0.45 mm, nylon
Flasks: 100 mL, brown

Reagents
0.1 M citric acid solution
Dissolve 10.5 g citric acid in 500 mL de-ionized water.

0.2 M sodium phosphate, dibasic solution
Dissolve 14.2 g sodium phosphate, dibasic in 500 mL de-ionized water.

McIlvaines Buffer: mix 445.5 mL of 0.1 M citric acid solution with 54.5 mL 0.2 M sodium phosphate, dibasic solution.

Elution Buffer: mix 5 mL of 25 % ammonia with 50 mL of ethyl acetate and 45 mL methanol, make fresh.

TMPD Solution: dissolve 50 mg of TMPD in methanol and make it to 50 mL.

Mobile Phases
Mobile Phase A: dissolve 0.39 g ammonium acetate in 900 mL de-ionized water, adjust the pH using acetic acid to pH 4.5 + 0.1, add de-ionized water to make the final volume 1000 mL, filter with 0.45 mm filters.

Mobile Phase B: add 1 mL formic acid to 1000 mL acetonitrile.

Internal Standards: take about 5 mg of isotope labeled malachite green and 5 mg of isotope labeled leucomalachite green, measure the weight accurately and then dissolve them in acetonitrile separately, then use acetonitrile to make the final volume 50 mL. Store these standards in the dark at -20 °C. Mix the standards and dilute them to 100 ng/mL with acetonitrile before using.

Standards: take about 5 mg of malachite green and about 5 mg leucomalachite green, measure the weight accurately, and then dissolve them in acetonitrile separately. Use acetonitrile to make the final volume 50 mL. Store these standards in the dark at -20 °C. Mix the standards and dilute them to 100 ng/mL with acetonitrile to make the standards stock. Make fresh standard by dilute the stock with 50 % acetonitrile to 0.5-10.0 ng/mL.

Sample Preparations
Sample extraction: cut sample into small pieces and homogenize it with the homogenizer. Take about 1 g homogenized sample, measure the weight accurately and put it in a plastic centrifugation tube. Add 50 mL internal standard solution, 50 mL TMPD solution, and 10 mL of McIlvaines buffer: acetonitrile (1: 1, v/v). Votex 45 seconds, then centrifugation at 2400 rpm for 20 minutes. Transfer the supernatant to a clean tube. Add 5 mL of McIlvaines buffer: acetonitrile (1 : 1, v/v) to the pellet, votex 45 seconds, then centrifugation at 2400 rpm for 20 minutes. Transfer the supernatant and add it to the supernatant from the first extraction.

Purification: prepare the Bond Elut Plexa PCX cartridge with 2 mL methanol, then 2 mL de-ionized water and then 2 mL McIlvaines buffer. Load supernatant sample from the extracted sample above on the pre-treated Bond Elut Plexa PCX cartridge, then add 0.1 N hydrochloric acid, wash twice with 2.5 mL de-ionized water. Dry the cartridge by vacuum, wash with 3 mL 50 % methanol, then 3 mL n-hexane. Dry the cartridge by vacuum for 5 min. Add 5 mL of elution buffer, collect the elute, dry the elute at 50 °C with nitrogen gas.

Add 1.0 mL of 50 % acetonitrile to the dried pellet, votex to dissolve the pellet, filter the solution through a 0.22 mm filter.

Standard Curve: spike the standard solutions to blank, prepare samples as before. Perform LC/MS according to the following conditions. Draw the standard curves by using the peak areas ratio of internal standards of malachite green and leucomalachite green and the correspondent concentrations of malachite green and leucomalachite green.

Conditions for LC/MS Testing
Mobile Phase: mixing mobile phase A and B with the ratio (v/v) and gradient below:

Time (min)	A (%)	B (%)
0.0	50	50
1.0	0	100
4.0	0	100
4.5	50	50
8.0	50	50

Flow Rate: 0.3 mL/min
Capillary Voltage: 2.5 kV
Ion Source Temperature: 100 °C
Desolvation Temperature: 400 °C
Detection Mode: multiple reaction monitoring (MRM) mode

Detection ion, cone voltage and collision energy are as follows:

Analyte	Mother Ion (m/z)	Daughter Ion ((m/z)	Cone Voltage (V)	Collision Energy (eV)
MG	329 329	313 208	60 60	35 37
LMG	331 331	239 316	40 40	30 24
MG-d5	334	318	40	38
LMG-d5	336	239	55	30

Quantitation ions: MG m/z 313 LMG m/z 239

Identification and Quantitation
Inject 20 mL of each sample and standards solutions to LC/MS. Use the previous conditions to perform the test. Using the peak areas and retention time of sample and standards, and multiple reaction monitoring mode for relative ion abundance*. Calculate the MG and LMG (ppb) in the sample by using the following formula:

$$\text{MG or LMG in sample (ppb)} = \frac{CxV}{M}$$

C = sample MG or LMG concentration (ng/mL) sample from standard curve
V = final volume of sample (mL)
M = sample weight (g)

* relative ion abundance range according to European 2002/657/EC rule:

Relative Ion Abundance (%)	Range %
>50	±20
>20-50	±25
>10-20	±30
</=10	±50

The detection limit for both MG and LMG using this method is: 0.5 ppb

If there is any known interference in the matrix, more discussion is needed.

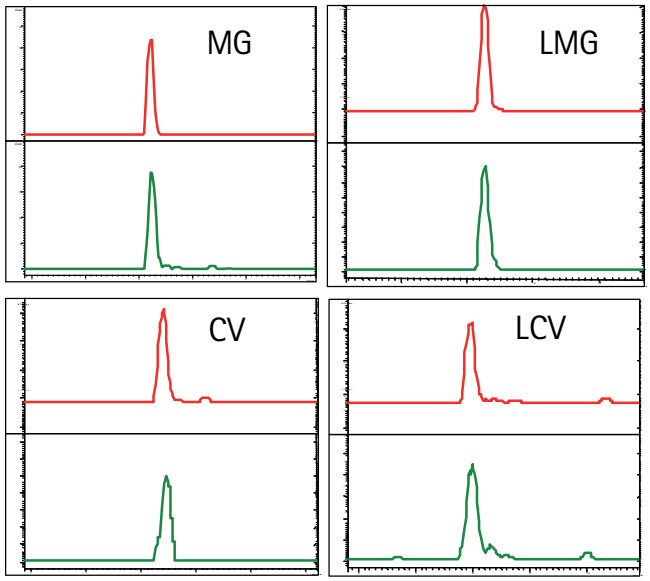

Figure 2. Chromatogram of each component at 1 ppb.

Calibration Curves Report

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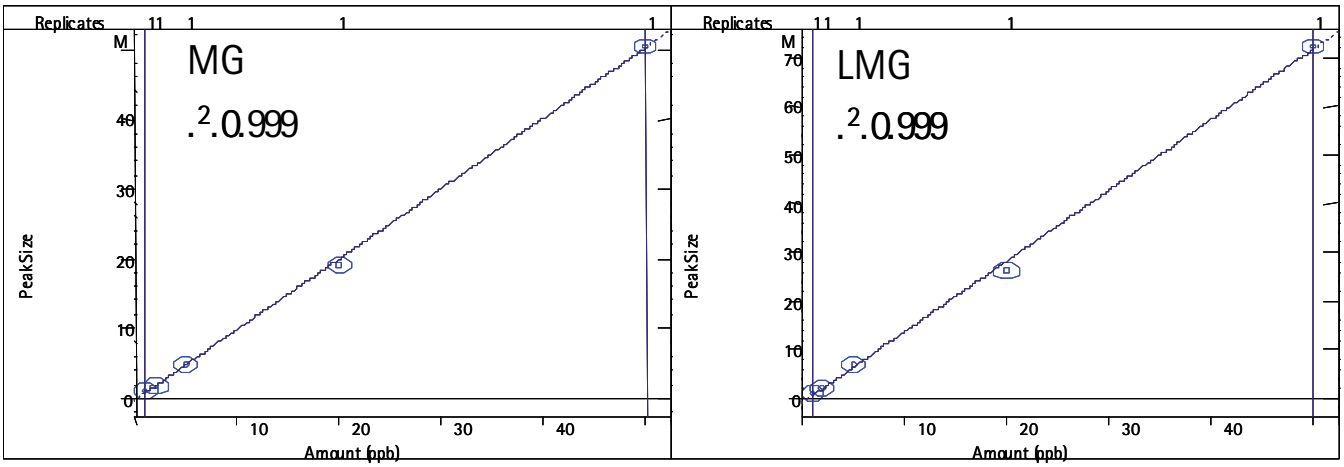
Method:	...nws \data\320lcms \malachite green \report\mcg_cv_mrm_r.mth		
Recalc Method:	...g_cv_mrm_r.mth	Last Calibration:	2008/02/07 4:47
Sample List:	N/A	Cmpd. Table Updated:	2008/02/19 11:55
Sequence:	N/A	Detector:	Quad Mass Spec
MS Workstation (Demo)		Workstation Version:	Version 6.9
Peak Measurement:	Area	Calibration Type:	External Standard Analysis

MG

Curve Fit: Linear, Origin: Ignore, Weight: None
Resp. Fact. RSD: 7.741%, Coeff. Det.(r2): 0.999480
y= +1.0100e+6x -3.1090e+5

LMG

Curve Fit: Linear, Origin: Ignore, Weight: None
Resp. Fact. RSD: 17.20%, Coeff. Det.(r2): 0.998654
y= +1.4526e+6x -9.8208e+5



CV

Curve Fit: Linear, Origin: Ignore, Weight: None
Resp. Fact. RSD: 17.63%, Coeff. Det.(r2): 0.999792
y= +1.3351e+6x -5.9818e+5

LCV

Curve Fit: Linear, Origin: Ignore, Weight: None
Resp. Fact. RSD: 24.08%, Coeff. Det.(r2): 0.998407
y= +6.5831e+5x -6.3367e+5

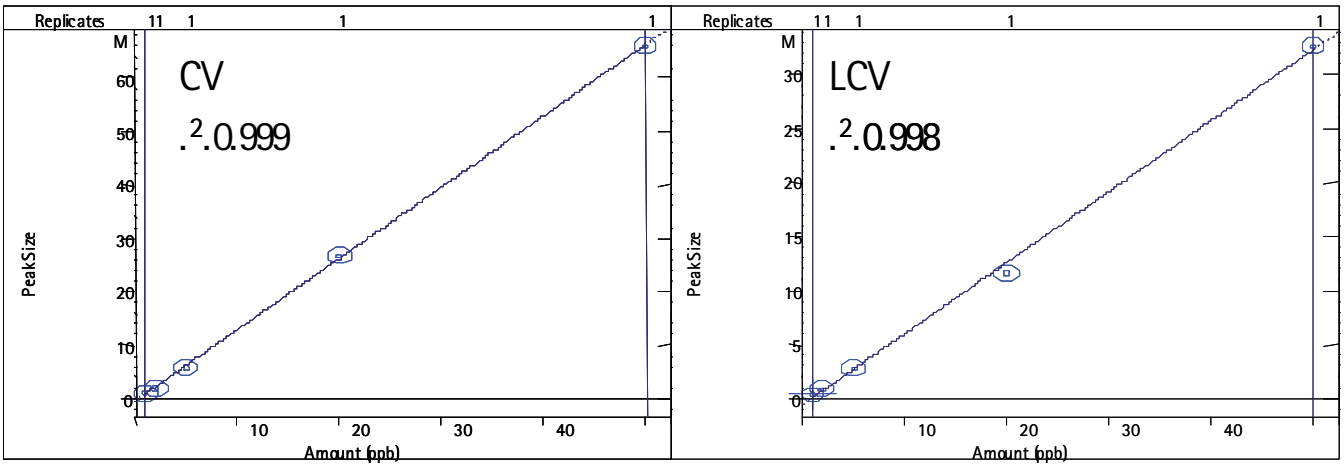


Figure 3. Linearity curves for each component, 1.50 ppb.

Conclusion

Malachite green (MG) and crystal violet (CV), and their metabolites leuco-malachite green (LMG) and leuco-crystal violet (LCV), are measured simultaneously. By the method required by the Japanese government the detection limit of MG is 0.002 ppm (with the extraction solution 0.01 ppm), and LMG is 0.01 ppm. Following this method, each component is well detected even at 1 ppb, which is a level of less than 1/10 of the required detection limit. In addition, linearity of each component is excellent.

These data represent typical results.
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