Application Note: 385

LC-MS/MS Determination of Malachite Green and Leucomalachite Green in Fish Products

Tao Ding[1], Jingzhong Xu[1], Bin Wu[1], Huilan Chen[1], Chongyu Shen[1], Fei Liu[2], and Kefei Wang[3]

[1] *Food Laboratory, APFIC, Jiangsu Entry-Exit Inspection and Quarantine Bureau (JSCIQ), Nanjing, China*
[2] *Thermo Fisher Scientific, Shanghai, China*
[3] *Thermo Fisher Scientific, San Jose, CA, USA*

Key Words

- Hypersil GOLD™
- Surveyor™ HPLC
- TSQ Quantum Discovery MAX™
- Food Safety
- H-SRM

Introduction

Malachite Green (MG, see Figure 1), a triphenylmethane dye, is an effective and inexpensive fungicide used in aquaculture, particularly in Asian countries. During metabolism MG reduces to Leucomalachite Green (LMG), which has been shown to accumulate in fatty fish tissues.

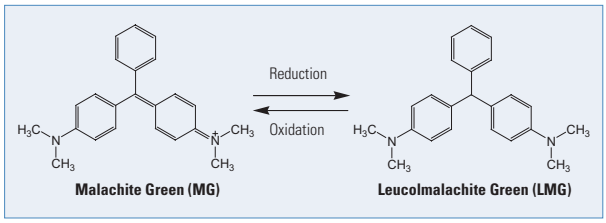

Figure 1: Structure and Conversion of Malachite Green and Leucomalachite Green

Both MG and LMG have demonstrated putative carcinogenic activity, and thus have been banned for use in aquaculture by both the U.S. FDA and European Union (EU). But trace levels of MG and LMG residues continue to be found in fish products. In a 2005 report,[1] malachite green was found in 18 out of 27 live eel or eel products imported from China to Hong Kong local market and food outlets, resulting in a government recall of all remaining products to be destroyed.

Based on European Commission decision 2002/657/EC, an analytical test method to detect MG and LMG must have a Minimum Required Performance Limit (MRPL) of 2 µg/kg of total malachite greens (MG+LMG) in fish muscle. Detection of MG and LMG has been reported by using UV-Vis, fluorescence spectrometry and mass spectrometry coupled to HPLC separation. Among these detection techniques, the sensitivity and selectivity are poor with UV-Vis, and the fluorescence detection requires a post-column oxidation (e.g. with lead oxide) to convert LMG to MG. Only mass spectrometry allows for detection of both LMG and MG without post-column oxidation, and with superior sensitivity and selectivity.[2]

In this work, we report an LC-MS/MS method to detect MG and LMG in roasted eel meat using a triple quadrupole mass spectrometer operated in highly selective reaction monitoring (H-SRM) mode. The method is sensitive and selective, and has been validated for routine detection of < 0.5 µg/kg of MG+LMG. Moreover, we demonstrate the capability of using H-SRM to reduce the chemical noise in complex sample matrices to improve detection of ultra-low level MG and LMG.

Experimental

Chemicals and Reagents

All chemicals were of reagent grade or better. MG oxalate salt and LMG were from Sigma-Aldrich (St Louis, MO, USA), and d_6-LMG from WITEGA (Berlin, Germany).

Sample Preparation

Extraction:

1. To 5.00 g of homogenized roasted eel meat, add 50 µL 1 µg/mL of d_6-LMG as internal standard (ISTD), 1 mL 0.25 g/L hydroxylamine hydrochloride (NH_2OH-HCl), 1 mL 0.05 mol/L *p*-toluenesulfonic acid, 2 mL 0.1 mol/L NH_4Ac-HAc buffer (pH 4.5), and 40 mL acetonitrile.
2. Homogenize for 2 min.
3. Centrifuge the mixture at 3000 rpm for 3 min.
4. Collect the supernatants into a 250-mL separation funnel.
5. Extract the meat once more with 20 mL acetonitrile.

Liquid-Liquid Extraction:

1. To the acetonitrile crude extract in the separation funnel, add 30 mL of dichloromethane (DCM) and 35 mL DI water, shake for 2 min.
2. Collect the DCM.
3. Extract the aqueous phase one more time with 20 mL DCM.
4. Evaporate the combined DCM solvent to dryness, and reconstitute in 3 mL of formic acid/acetonitrile (2:98).

Solid Phase Extraction (SPE):

1. Condition the Oasis 60 mg/3 cc MCX cartridge (Waters, Milford, MA, USA) with 3 mL acetonitrile, and 3 mL 2% v/v formic acid aqueous solution.
2. Load the sample (at ~0.2 mL/min).
3. Wash with 2 mL formic acid:acetonitrile (2:98) and 6 mL of acetonitrile.
4. Elute with 4 mL NH_4Ac (5 mol/L and pH 7)/MeOH (5:95).
5. Evaporate the MeOH at 45 °C under reduced pressure
6. Dilute to 1.0 mL with initial mobile phase of water (0.1% v/v formic acid)/MeOH (70:30)
7. Filter with a 0.45 µm syringe filter before injection to LC-MS.

Note: For very fatty roasted eel tissues, prior to the SPE with the MCX, the extracts after liquid-liquid extraction were cleaned up with a Superclean 60 mg/3 cc LC-Alumina N cartridge (Waters, Milford, MA, USA):

1. Condition the cartridge with 3 mL acetonitrile
2. Load the sample, collect the elute
3. Wash with 3 mL acetonitrile, collect the elute to be combined with elute in (2)
4. Add 120 µL of formic acid to the combined elute.

Chromatography Conditions

HPLC:	Surveyor HPLC (Thermo Fisher Scientific, Waltham, MA, USA)
Column:	Hypersil GOLD CN 50 × 2.1 mm 5 µm
Mobile Phase:	A: Methanol
	B: Water with 0.1% v/v Formic Acid

Gradients:	Time (min)	A%
	0.0	30%
	2.0-6.0	90%
	6.1	30%
	10.0	30%

Flow Rate:	220 µL/min
Injection volume:	10 µL

Mass Spectrometry Conditions

Mass Spectrometer:	TSQ Quantum Discovery MAX (Thermo Fisher Scientific, Waltham, MA, USA)
Source:	ESI+, 4000 V
Sheath Gas:	40 unit
Auxiliary Gas:	5 unit
Capillary Temperature:	350°C
Source CID:	-10 V
Q1 Peak Width (FWHM):	0.7 Da (0.2 Da for H-SRM)
Q3 Peak Width (FWHM):	0.7 Da
Collision Gas:	Ar (1.5 mTorr)
SRM Transitions:	See Table 1
Scan Time:	0.1 s

	Precursor Ion	Product Ion (Collision Energy)
MG (M*)	329.1	313 (33)* 208 (48)
LMG (MH^+)	331.3	239 (31)* 316 (18)
d_6-LMG (MH^+)	337.2	240 (30)

Table 1: SMR Transitions and Collision Energy Values for MG and LMG

Results and Discussion

Eel meat tissues are in general fatty, and the roasted eel meat contains additional cooking oil and flavor chemicals, making the sample matrix complicated. The extraction of MG and LMG from roasted eel meat involves sample extraction, liquid-liquid extraction, and one or two steps of SPE clean up. A similar method using the liquid-liquid extraction (with DCM) and one step SPE clean up (with SCX) were also reported for analysis of MG and LMG in both the raw and the "processed eel products."[3] For extraction of MG and LMG in other raw fish meats, the procedure could be simplified. For example, Roudaut et al. have recently reported the following method without using the SPE for salmon, trout, tilapia and catfish:[4]

Use 2 g of homogenized sample
Add 200 µL standard solution
(for spike experiment only)
Add 200 µL ISTD solution 20 ng/mL
Add 600 µL Water (800 µL for unknown)
Add 2 mL Hydroxylamine HCl 5 g/L
Stir the mixture for 10 min
Add 8 mL acetonitrile
Stir 10 min at 100 rpm
Centrifuge for 5 min
Filter on 0.45 um
Inject 20 µL to a TSQ Quantum™

Figure 2 shows the comparison of SRM and H-SRM chromatograms of a matrix matched standard (i.e., standard spiked into a blank roasted eel extract sample *after* sample preparation) containing 0.02 pg/µL (0.2 pg on-column) MG and 0.1 pg/µL (1 pg on-column) LMG and with 1 pg/µL ISTD. As shown, with H-SRM, signal-to-noise (S/N) ratios have improved significantly from 2-5 to 20-25. Note that the S/N improved despite the absolute signal (measured by the peak areas) decreasing by approximately half, indicating that the gains in S/N are from eliminating noise (isobaric interferences) in the sample matrix. The instrument detection limit in the current matrix is thus estimated to be 0.1 pg for MG and 0.5 pg for LMG with H-SRM based on 10× S/N. These detection limit values, corresponding to 0.004 µg/kg and 0.02 µg/kg for MG and LMG in meat tissues, respectively, have far exceeded our current requirement to detect <0.5 µg/kg of MG+LMG in roasted eel meat.

The response linearity was evaluated over the range of 0.05-8.0 µg/kg using matrix matched standard solutions. The correlation coefficients obtained are >0.99 (weight factor = 1/X). Figure 3 shows the representative calibration curves.

The analytical method was validated by analyzing fortified roast eel samples at 1, 2 and 5 µg/kg levels for both LG and LMG, corresponding to 0.5×, 1×, and 2.5× MRPL, respectively. Seven replicates were performed at each level. The results are summarized in Table 2. Excellent recovery values of 90-106% were obtained with RSD% ranging from 3.7 to 11%.

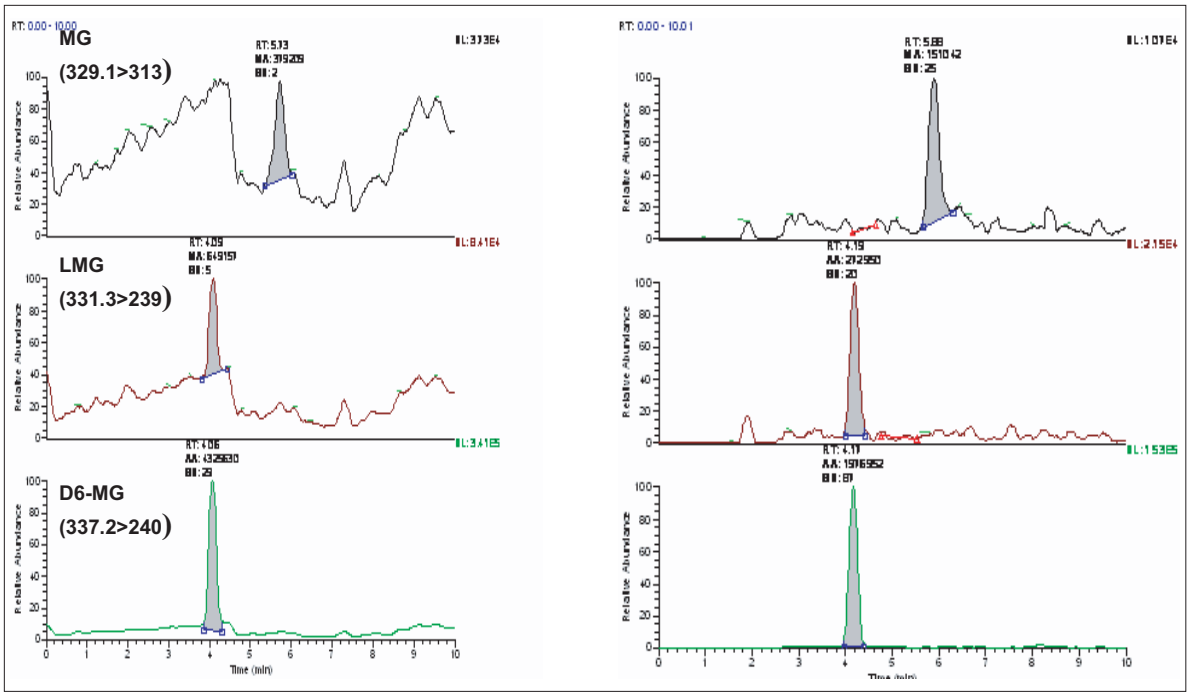

Figure 2: Comparison of SRM (left) and H-SRM (right) Chromatograms of a Matrix Matched Standard Containing 0.02 pg/µL MG and 0.05 pg/µL LMG (10 µL injection).

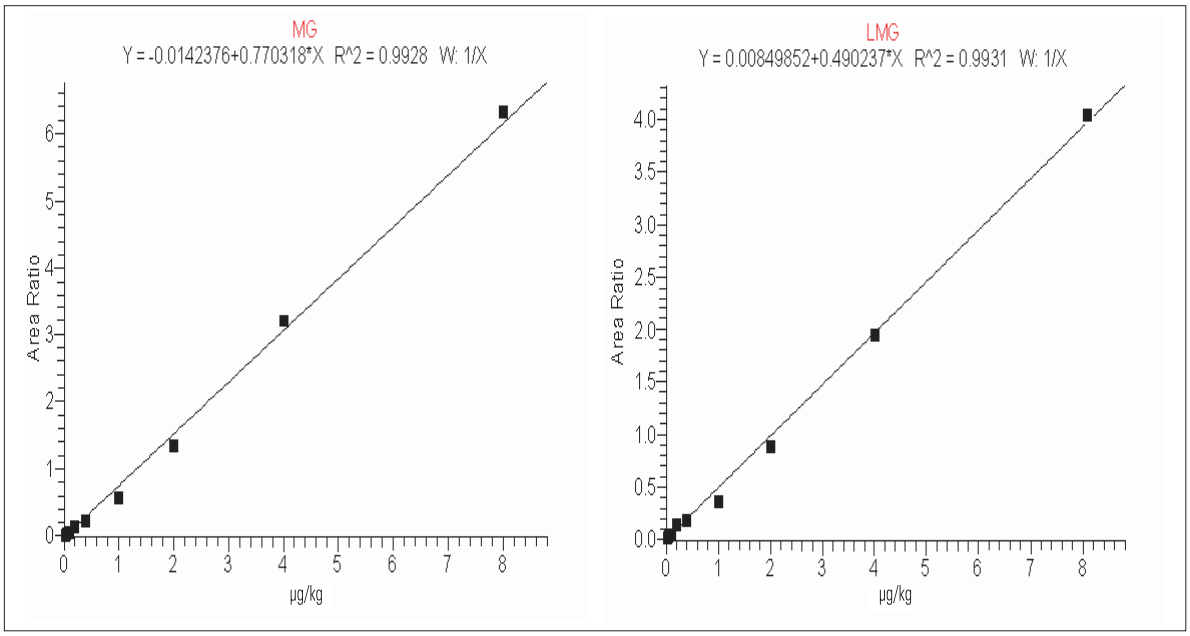

Figure 3: Representative calibration curves for MG and LMG with matrix matched standard solutions.

Spike (µg/kg)	1.0	2.0	5.0
MG	95 (5.8)	101 (3.7)	90 (7.5)
LMG	106 (7.0%)	94 (11)	92 (4.7)

Table 2: Recovery% (RSD%) of MG and LMG (ISTD Corrected) in Roasted Eel Meat (n=7)

Conclusions

A highly sensitive and selective LC-MS/MS method using the TSQ Quantum Discovery MAX has been developed for determining Malachite Green and Leucomalachite Green in roasted eel meat. The method shows excellent linearity (0.05 to 8.0 µg/kg), accuracy (90-106% recovery) and reproducibility (4-11% RSD), far exceeding the EU's requirement of MRPL of 2 µg/kg of MG+LMG. The method has been implemented at the JSCIQ lab for routine monitoring of <0.5 µg/kg (MG+LMG) in roasted eel and other fish products (with variations of sample preparation procedures).

Highly selective reaction monitoring (H-SRM) has been shown to reduce the chemical noise effectively in the complicated sample matrix, which should be useful to further improve the method sensitivity and specificity (i.e., to eliminate both false positive and false negative) in support of enforcement of a "zero tolerance" policy toward the use of MG and LMG for aquaculture.

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