



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



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Overview

Purpose: To develop a method for the analysis of malachite green and leucomalachite green residues in roast eel flesh by LC-MS-MS with LOQs below 0.5 ug/kg

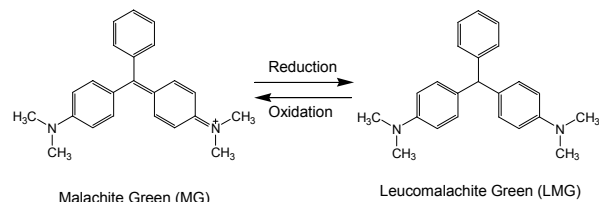
Methods: The MG and LMG in roast eel were extracted with ammonium acetate and methylene chloride, followed by solid phase extraction cleanup. D6-LMG was used as the internal standard. The MG and LMG were separated by HPLC followed by detection with a triple quadrupole mass spectrometer operated in selected reaction monitoring (SRM) mode.

Results: The LOQ of the method is well below 0.5 ug/kg, as the instrument detection limit (IDL) as low as 0.1 pg for MG and 0.5 pg for LMG can be achieved with the use of high resolution SRM (H-SRM) by setting Q1=0.2 Da.

The method has a linearity range of 0.05-8 ug/kg. Recovery values for spikes at 1, 2 and 5 ug/kg are in the range of 90-106% (RSD%=4-11%).

Introduction

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



Both MG and LMG have demonstrated putative carcinogenic activity, and thus have been banned for use in aquaculture by both the US FDA and European Union (EU). But trace levels of LMG residue continues to be found in fish product. In a 2005 report, 18 out of 27 eel products exported from Guangdong Province of China to the Hong Kong local market were found to contain LMG, resulting in the destruction of all of the eel products.

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 ug/kg of MG+LMG in fish muscle. Detection of MG and LMG have been reported by using UV-Vis, fluorescence spectrometry and mass spectrometry coupled to the HPLC separation. While sensitivity and selectivity are poor with UV-Vis detection, post-column oxidation (e.g. with lead oxide) to convert LMG to MG is required for fluorescence detection. Mass spectrometry allows for detection of both LMG and MG without post-column oxidation and with superior sensitivity and selectivity.

In this work, we report an LC-MS-MS method to detect MG and LMG in roast eel flesh using a triple quadrupole mass spectrometer operated in H-SRM (Highly-Selective Reaction Monitoring) mode. The method is sensitive and selective, and has been validated for use to detect < 0.5 ug/kg of MG+LMG. Moreover, we demonstrate the capability of using H-SRM to reduce the chemical noise to improve detection of ultra-low level MG and LMG.

Methods

Chemicals and Reagent: All chemicals were of reagent grade or better. MG oxalate salt and LMG were from Sigma-Aldrich (St Louis, MO, USA), and d₆-LMG from WITEGA (Berlin, Germany).

Extraction: To 5.00 g of homogenized roast eel flesh, add 50 uL 1 ug/mL of d₆-LMG, 1mL 0.25 g/L NH₂OH-HCl, 1 mL 0.05 mol/L p-toluenesulfonic acid, 2 mL 0.1 mol/L NH₄Ac-HAc buffer (pH 4.5), and 40 mL CH₂CN, followed by additional homogenization for 2 min. Centrifuge at 3000 rpm for 3 min, collect the supernatants into a separation funnel. Extract the solid meat flesh with 20 mL CH₂CN. Combined the supernatants in the funnel, to which 30 mL of CH₂Cl₂ and 35 mL DI Waters were added. Perform the liquid-liquid extraction, the organic layer were collected. Extract the aqueous phase one more time with 20 mL CH₂Cl₂. Combined the CH₂Cl₂ extracts and evaporated to dryness, and reconstitute in 2% v/v Formic Acid:MeOH.

Solid Phase Extraction: OasisTM 60 mg/3 cc MCX cartridge (Waters, Milford, MA, USA) was used. The cartridge was activated by 3 mL CH₂CN, followed by 2% v/v formic acid aqueous solution. Load the sample, wash with 2 mL 2% v/v Formic acid:CH₂CN and 6 mL of CH₂CN. Elute MG and LMG with 2% v/v NH₄Ac (5 mol/L and pH 7):MeOH. The eluent was concentrated at 45 °C under reduced pressure to 1.0 mL.

Note: For very fatty eel flesh samples, the extracts were pre-treated with Superclean 60 mg/3 cc LC-Alumina N cartridge (Waters, Milford, MA, USA) to remove the fat before SPE with MCX.

Chromatography Conditions:

HPLC: SurveyorTM Module HPLC (Thermo Electron)

Column: Hypersil GoldTM CN 50 mm x 2.1 mm 5 u

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 uL/min

Injection volume: 10 uL

Mass Spectrometry Conditions:

Mass Spectrometer: FinniganTM TSQ Quantum Discovery MaxTM (Thermo Electron)

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: 2 SRM each for MG and LMG (See Table 1)

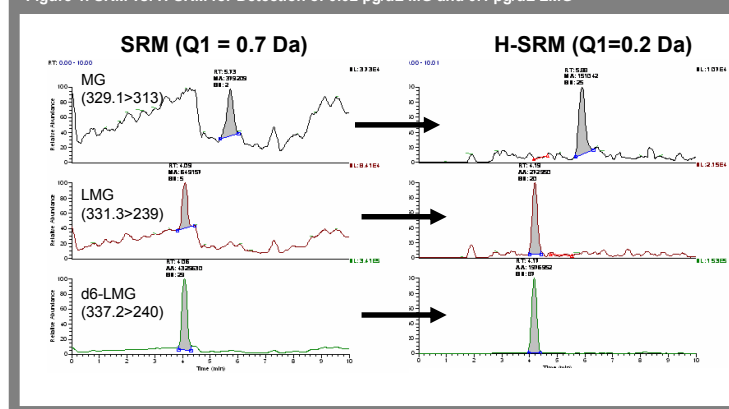
	Precursor Ion	Product Ion (Collision Energy)
MG (M ⁺)	329.1	313 (33)* 208 (48)
LMG (M+H ⁺)	331.3	239 (31)* 316 (18)
d ₆ -LMG (M+H ⁺)	337.2	240 (30)

* SRM transition for quantitation

Results & Discussion:

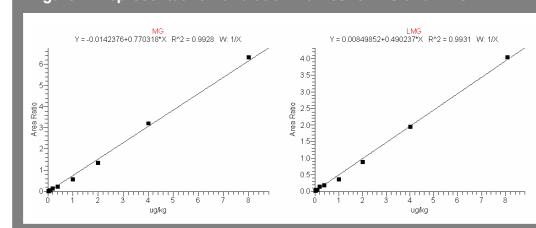
Figure 1 shows the comparison of SRM and H-SRM chromatograms of a standard solution containing 0.02 pg/uL (0.2 pg on-column) MG and 0.1 pg/uL (1 pg on-column) LMG and with 1 pg/uL IS. As shown, with H-SRM, signal-to-noise (SN) ratios have improved 10 fold from 2-5 to 20-25. The Instrument Detection Limit (IDL) is estimated to be 0.1 pg for MG and 0.5 pg for LMG with H-SRM based on 10x S/N. These IDL values far exceed our current requirement to detect 0.5 ug/kg MG+LMG in roast eel flesh samples.

Figure 1. SRM vs. H-SRM for Detection of 0.02 pg/uL MG and 0.1 pg/uL LMG



Linearity was evaluated in the range of 0.05-8.0 ug/kg using the matrix matched standard solutions. The correlation coefficients obtained are > 0.99 (weight factor = 1/X). Figure 2 shows the representative calibration curves.

Figure 2. Representative Calibration Curves for MG and LMG



The analytical method was validated by analyzing the fortified roast eel flesh samples at 1, 2 and 5 ug/kg levels, corresponding to 0.5x, 1x and 2.5x 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%.

Spike (ug/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)

Conclusions

A highly sensitive and selective LC-MS/MS method has been developed for determining Malachite Green and Leucomalachite Green in roast eel flesh. The method has shown good response linearity (0.05 to 8.0 ug/kg), accuracy (90-106% recovery) and reproducibility (4-11% RSD), far exceeding the EU requirement of MRPL of 2 ug/kg.

The high resolution SRM (H-SRM) has been shown to further reduce the chemical noise for detecting ultra-low level of these drug residues, which is useful for us to develop more sensitive analytical method in support of enforcement of a "zero tolerance" policy toward the use of MG and LMG for aquaculture in China.

References

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