Extraction of Malachite Green, Crystal Violet and their Leuco Metabolites From Salmon Using QuEChERS and EVOLUTE CX for LC-MS-MS Analysis

Introduction

This application note describes the extraction of malachite green, leuco malachite green, crystal violet (gentian violet), leuco crystal violet (leuco gentian violet) and brilliant green from oily fish tissue using enhanced dispersive SPE followed by EVOLUTE CX clean up.

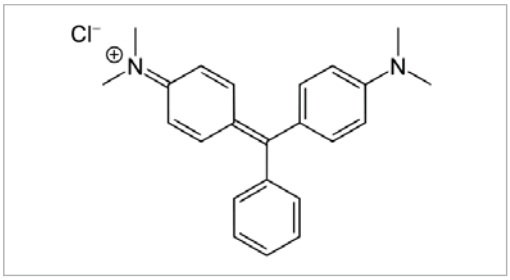

Figure 1. Structure of Malachite green

Analytes

Malachite green, Leuco malachite green, Crystal violet, Leuco crystal violet and Brilliant green

Malachite green (MG) and crystal (gentian) violet (CV) are triphenylmethane dyes used in aquaculture as fungicides and ectoparasiticides. They are rapidly metabolised to the leucomalachite green (LMG) and leucocrystal (gentian) violet (LCV) metabolites that persist in fish tissue. MG and CV are banned in many countries due to their mutagenicity and carcinogenicity, but may still be used illegally.

Monitoring methodologies must be able to detect the compounds at 1 ng/g (US) or 2 ng/g (EU). In order to meet these LODs for complex matrices such as oily fish, and the low UV absorbance of the leuco forms, LC-MS-MS is the analytical method of choice. This application note focuses on ease of use when compared to other methods, and describes a QuEChERS based approach incorporating the use of aluminium oxide in enhanced dispersive SPE to reduce the levels of fat in the final extract. Additional cleanup is provided by polymeric EVOLUTE SPE columns, and the resulting extract demonstrated low ion suppression. Analyte recoveries of 60-105%, with low rsds, are achieved. The method is also applicable to Brilliant green

Sample Preparation Procedure

Sample pre-treatment: Weigh 1.0 g of fish (salmon) tissue. Add Hydroxyl amine hydrochloride (0.25 mg/mL, 200 µL), p-toluene sulfonic acid (1 M , 20 µL), ammonium acetate (1 M, pH 4.5, 20 µL) and acetonitrile (6 mL). Add 100 ng/mL internal standard solution 20 µL and homogenize.

Phase separation: Transfer homogenate quantitatively to a QuEChERS extraction tube (**Q0020-15V**, containing 4 g magnesium sulfate, 1 g sodium chloride, 1 g sodium citrate and 0.5 g sodium citrate sesquihydrate). Rinse the homogenization beaker with water (2 x 1 mL) which also is added to the centrifuge tube. Vortex for 30 s. Centrifuge for 1 min at 3000 g to separate the two phases formed.

Dispersive SPE: To a 15 mL centrifuge tube containing 1 g neutral aluminium oxide and 0.2 g magnesium sulphate, transfer 4 mL of the top acetonitrile layer. Vortex for 30 s. Centrifuge for 1 min at 3000 g.

EVOLUTE configuration: EVOLUTE CX 50 mg/3 mL, part number **611-0005-B**

Conditioning: Apply acetonitrile (2 mL) followed by 5% acetic acid in acetonitrile (2 mL).

Sample loading: Transfer 2 mL of the supernatant from the dSPE tube to the CX column at a flow rate of 3 mL / min.

Interference elution 1: Apply 2 mL of 5% acetic acid in acetonitrile.

Interference elution 2: Apply 2 x 3 mL acetonitrile.

Analyte elution: Elute analytes with 2% ammonium hydroxide in acetonitrile (3 mL).

Post-extraction: Evaporate the elution extract to dryness (50 ºC for 90 min in centrivap). Reconstitute in 70% mobile phase A / 30% mobile phase B.

HPLC Conditions

Instrument: Shimadzu HPLC system consisting of a DGU-20A3 vacuum degasser, two LC-20AD pumps, and a SIL-20AC autosampler.

Column: Supelco Ascentis Express C18, 50x3.0 mm, dp = 2.7 µm or Waters Xselect CSH C18, 50x3.0 mm, dp =2.5 µm.

Temperature: Ambient.

Flow rate: 0.4 mL/min

Injection volume: 10 µL.

Mobile phase: A= 0.25% formic acid in 0.1 M ammonium acetate (pH 4.5).

B= 0.5% formic acid in acetonitrile.

Gradient:

Time (min)	%B
0	30
0.5	30
7	95
11	95
11.1	30
13	30

Mass Spectrometry Conditions

Instrument: AB Sciex API3200 triple quadropole mass spectrometer with an electrospray interface. Positive ions were acquired in the multiple reaction monitoring mode (MRM).

Desolvation Temperature: 550 °C

Curtain gas: 20 psi

Collision gas: 4 psi

Ion spray voltage: 4.5 kV

Ion source gas 1: 50 psi

Ion source gas 2: 30 psi

Table 1. MRM transitions for all analytes

Analyte	Q1 (m/z)	Q3 (m/z)	Declustering potential (V)	Entrance potential (V)	Collision entrance potential (V)	Collision energy (V)	Collision exit potential (V)
MG	329.4	313.3	68	6	16	46	26
LMG	331.4	239.1	58	5	15	40	20
CV	372.4	356.4	78	4	16	52	28
LCV	374.4	358.4	57	5	16	40	30
BG	385.4	341.4	70	5	18	50	28
MG-d5	334.4	318.3	68	6	16	46	26
LMG-d5	336.4	239.1	58	5	15	40	20
Dwell time = 100 ms							

Results

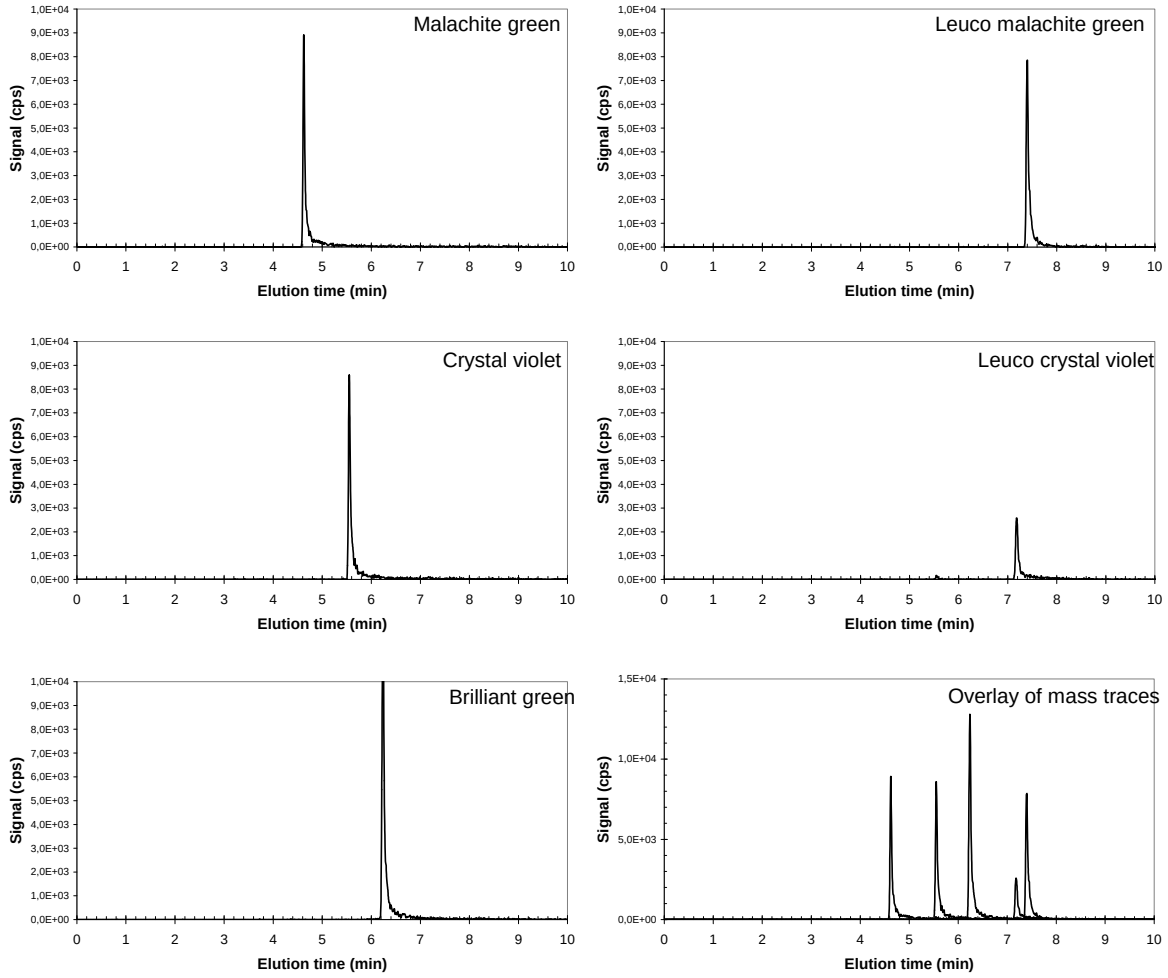

Figure 1. LC-MS chromatogram from the analysis of a mix of the five compounds, malachite green, leuco malachite green, crystal violet, leuco crystal violet, and brilliant green. Concentration of each component is 1 ng/mL.

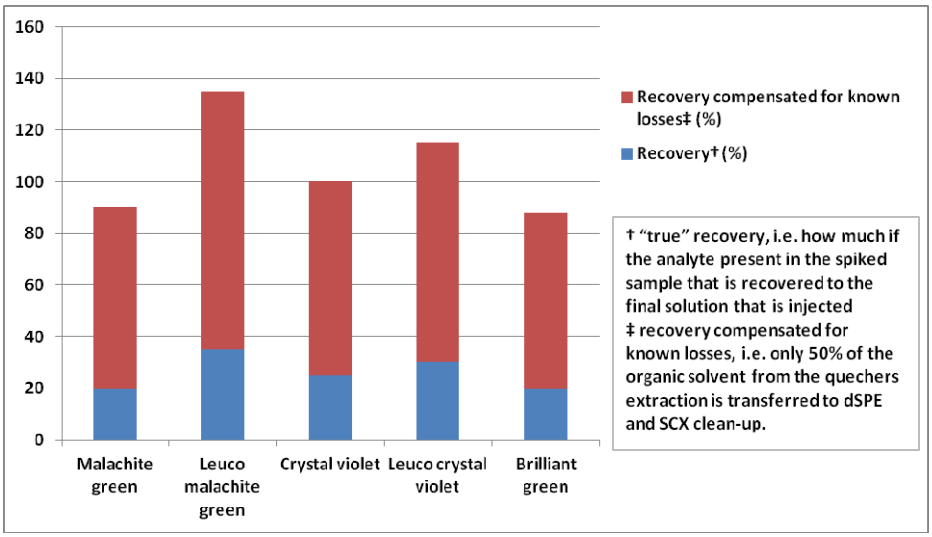

Figure 2. % Recoveries for all analytes both true analyte recovery and adjusted based upon loss from initial clean up

Table 2. Ion suppression when comparing spiked salmon extracts with spiked pure solvent and recovered concentration of dyes from 5 ng/g spiked sample

	Malachite green	Leuco malachite green	Crystal violet	Leuco crystal violet	Brilliant green
Ion Suppression	4%	3%	-19%	-6%	1%
Recovered concentration from a 5ng/g spike of homogenized salmon (ng/g)	5.3	5.6	8.1	4.8	5.6

Ordering information

Part number	Description	Quantity
611-0005-B	EVOLUTE CX 50 mg/3 mL	50
Q0020-15V	10 g QuEChERS AOAC 15 mL Extraction Tube	25
9714-0100	ISOLUTE AL-N Bulk (100 g)	100 g

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