Application of UPLC-MS/MS for Simultaneous Determination of Malachite Green and Metabolites Residues in Tilapia

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Abstract. In this work, a convenient, sensitive method for simultaneous monitoring of malachite green, leucomalachite green in tilapia samples was developed by ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The tilapia samples were extracted by acetonitrile, separated with acetonitrile-5mmol/L ammonium acetate as mobile phase, and then detected under ESI+ mode. The method showed a good linearity for all the analyts over the range of 0.1-100 μg kg⁻¹ with r²=0.998. And the detection limits both were 0.10 μg kg⁻¹ for Malachite green and leucomalachite green. Tests for recovery were made by addition of standards at four different concentration levels (0.5、2.0、10.0、20.0 μg kg⁻¹) to the blank sample. The recoveries of the four spiked concentrations were ranged from 81.5% to 104%, with the relative standard deviations lower than 5%. The sensitivity, accuracy and precision of this method were able to meet the requirements for malachite green and leucomalachite green residue analysis.

1. Introduction
Malachite green is an industrial triphenylmethane dye, which has been widely used in the aquaculture industry, because of its efficiency against parasitic infection and fungal in fish [1,2]. Malachite green residues in fish for quite a long time and can cause some side effects, such as high residual, high toxins, carcinogenic, mutation and teratogenic. Therefore, taking contaminated fish may cause serious health problems of human [3,4]. Normally, Malachite green will be reduced into leucomalachite green after administration, and potential carcinogenic, mutagenic, genotoxic, and teratogenic properties have been found in many animal species [3]. For this reason, the use of Malachite green is under strict scrutiny in many countries, and the administration of Malachite green is also illegal in China. Nevertheless, Malachite green is still largely used because it is a highly effective parasiticide and fungicide [5,6].

Numerous strategies including enzyme-linked immuno sorbent assay (ELISA), gas chromatography- mass spectrometry, high-performance liquid chromatography and liquid chromatography-mass spectrometry have been used for detection of Malachite green and
leucomalachite green in aquatic [7-12]. Among the variety strategies, ELISA appears to be a quick and sensitive method, and has been successful applied for detection of Malachite green. However, ELISA always shows false positive result and is mostly used for preliminary screening. Additionally, the use of high-performance liquid chromatography or gas chromatography-mass spectrometry for analysis of transformation or derivatization is rather tedious and time-consuming. In contrast, the detection of metabolites and the leuco-metabolites at regulated levels has been largely improved by using of liquid chromatography-mass spectrometry. With the development of this technology, ultra-high-performance liquid chromatography-mass spectrometry arises as an important tool for confirmation and determination.

The current study aims to develop a rapid UPLC-MS/MS method with high sensitivity, easy to operation, selectivity and fast to perform, for detection of Malachite green and leucomalachite green residing in tilapia. The advantage of using this method for analysis is due to its sensitivity, simplicity, high stability, and anti-interference performances.

2. Instruments, Reagents and materials

**Instruments:** ACQUITY UPLCTM-TQ UPLC-MS/MS (waters Corporation), IKA MS3 Vortex mixer (Germany), IKA T25 Homogenize (Germany), Milli-QA1 (Millipore), CR22GIII centrifuge (Japan-Hitachi), EE120H Ultrasoundscope (Elma).

**Reagents:** Malachite green, Leuco-malachite green, malachite green-D5(D5-MG), and leucomalachite green-D6 (D6-LMG) obtained from Dr. Ehrenstorfer were of chromatographic grade (purity≥98%). Acetonitrile and Ammonium acetate purchased from Spectrum and DIMA were of chromatographic grade. The stock solutions at a concentration of 100 mg L⁻¹ in acetonitrile were prepared, respectively. All working solutions were prepared by sequential dilution of the stock solutions with acetonitrile and kept at 4 °C.

**Materials:** Tilapia were obtained from aquatic market of Zhanjiang. All the samples were weighed and their bone, head and fat were removed, when reached the laboratory. Fresh Tilapia were separated, homogenised and stored at -18°C. The specimen with no detectable residues of the analytes were used as negative controls.

3. Analytical procedures

3.1. Instrument condition

The separation of MG and LMG on ACQUITY UPLC BEH C18 column (50×2.1mm, 1.7μm) was achieved using a gradient UPLC system (table 1), with acetonitrile and ammonium acetate solution (5 mmol L⁻¹) at a flow rate of 0.30 mL min⁻¹. The temperature of the column was 40°C, and the volume of the injection was 10.0 μL. The detection was carried out using ESI+ with a multiple reaction monitoring mode. The interface conditions were set as follows: the source temperature is 110°C, the capillary voltage is3.0 kV, the desolation temperature is 350°C, and the flow rates of desolation gas and cone are 800 L/h⁻¹ and 60 L h⁻¹, respectively. The parameters of MS/MS were listed in table 2.

| Time (min) | Flow rate (mL min⁻¹) | acetonitrile (%) | 5 mmolL⁻¹ ammonium acetate solution B (%) |
|------------|----------------------|-----------------|----------------------------------------|
| 0          | 0.30                 | 50              | 50                                     |
| 1.0        | 0.30                 | 80              | 20                                     |
| 2.0        | 0.30                 | 90              | 10                                     |
| 2.5        | 0.30                 | 80              | 20                                     |
| 3.0        | 0.30                 | 50              | 50                                     |
4. Results and discussion

4.1. Selection of extraction solvents

There are strict requirements on the use of solvent for residue analysis, such as high extraction recovery rates, matrix interference small impurities, economic, small environmental pollution, and so on. Malachite green and Metabolites are soluble in water, methanol and acetonitrile. The effects of methanol and acetonitrile extraction on fortified recovery rates were studied in a comparable manner in this study. Results showed that the extractions of acetonitrile can achieve high fortified recovery rates. The acetonitrile also brought high extraction efficiency and small impurity interferences. Therefore, acetonitrile was used as the extraction solvent for the following studies.

4.2. Selection of mobile phase

Two kinds of mobile phase, acetonitrile/ammonium acetate and methanol/water composition that with different ratio were selected. Results showed that when acetonitrile-5 mmol L⁻¹ ammonium acetate solution was used as the mobile phase, the chromatographic separation effect was the best, and the baseline was relatively stable. Moreover, lower noise was obtained under the acetonitrile-5 mmol L⁻¹ ammonium acetate 50:50 (v/v) condition, and Malachite green and Leuco-malachite green both had a higher signal to noise ratio and better chromatographic peak shape.

4.3. The linear range of the methods

The calibration curves for Malachite green and Lleuco-malachite green were obtained by plotting the peak area (y) versus concentration (x) of each analyte. The curves were expressed as y=0.6324x+0.0015 with a correlation coefficient of 0.9984 for Malachite green and y=0.5647x+0.0079 with a correlation coefficient of 0.9989 for Leuco-malachite green. The calibration curves were daily created from the peak area responded to standards with a concentration ranged from 0.1 to 100 µg kg⁻¹.
Table 3. Linear ranges, regression equation and correlation coefficient.

| Compound   | Linear range (μg kg\(^{-1}\)) | Regression equation | Correlation coefficient (%) |
|------------|--------------------------------|----------------------|----------------------------|
| MG         | 0.1-100                        | y=0.6324x+0.0015     | 0.9984                     |
| LMG        | 0.1-100                        | y= 0.5647x+0.0079    | 0.9989                     |

4.4. LOD of the methods

Based on three times of signal to noise ratio (S/N=3), the detection limits of the instrument were tested. The standard solution was added into the negative samples, and then pre-treated and analysed accorded to the method described above. The LOD for Malachite green and Leuco-malachite green were calculated via analysis of the negative samples that mixed with standard solution. The LOD based on three times of signal to noise ratio was 0.10 μg kg\(^{-1}\) for Malachite green and Leuco-malachite green.

4.5. Accuracy of the methods

To test its accuracy, negative samples were added in to the Malachite green and Leuco-malachite green standard solutions, respectively. The four fortification levels were analysed with six tests per sample. The recovery of the method was investigated using tilapia samples strengthened at 0.5、2.0、10.0、20.0 μg kg\(^{-1}\). Average recovery (n=6) of the analytes determined with three independent assays was shown in table 4, which was between 81.5% to 104% and with relative standard deviations below 5%.

Table 4. RSD of MG and LMG in samples

| Compound       | Spiked concentrations (μg/kg) | Mean measured concentrations (μg/kg) | Mean recovery (%) | RSD (%) |
|----------------|------------------------------|-------------------------------------|-------------------|---------|
| Malachite green| 0.5                          | 0.51                                | 102               | 4.8     |
|                | 2.0                          | 1.95                                | 97.5              | 4.3     |
|                | 10.0                         | 9.36                                | 93.6              | 3.7     |
|                | 20.0                         | 17.7                                | 88.5              | 4.1     |
| Leuco-malachite green | 0.5         | 0.52                                | 104               | 4.9     |
|                | 2.5                          | 1.85                                | 92.5              | 3.6     |
|                | 10.0                         | 9.06                                | 90.6              | 3.8     |
|                | 20.0                         | 16.3                                | 81.5              | 4.4     |

5. Conclusions

This study established a method for determination of Malachite green and Leuco-malachite green in tilapia. The results showed that this method has the advantages of easy to operate, high sensitivity and accuracy, and it can be used for detection of residues of Malachite green and Leuco-malachite green in tilapia.

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