

Detection and Temporal Variation of Malachite Green in the Sepang River Using Solid Phase Extraction and Spectrophotometry

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HISTORY

Received: 25th Sep 2024
Received in revised form: 25th Nov 2024
Accepted: 29th Dec 2024

KEYWORDS

Malachite green
Sepang River
Solid phase extraction
Spectrophotometric detection
Aquaculture pollution

ABSTRACT

Malachite green (MG) is still used at present to cure fish and prawn that have been infected with pathogenic agents in aquaculture and the ornamental fish sector. It is most used for its antifungal, antiparasitic, and antibacterial activities. The therapeutic and lethal dose is also fairly close, and there is a risk of overdoses when used for treatment. Moreover, MG is also emitted into the environment through the disposal of effluents from aquaculture farms, which may lead to aquatic environments being polluted. This study aimed to establish a method of detection of Malachite Green (MG) in environmental samples using solid phase extraction in conjunction with visible spectrophotometry. The calibration curve was linear ($R^2 > 0.99$) in the range of 0.1-1.5 mg/L, and the detection limits were 0.034 mg/L in deionized water and 0.082 mg/L in Sepang River water. Although these limits are not suitable for environmental analysis down to the parts per billion level, the water samples were first concentrated using a solid phase extraction column based on a biosorbent to enhance sensitivity by about a hundredfold. There were significant matrix effects, but robust sample preparation was used to reduce them. The concentrations of MG varied during the day and were highest at midday, which might have been because of higher aquaculture or industry activity at that time. Even though the levels were below the recommended limits for aquaculture products, the existence of MG is hazardous to the environment and human health because of its carcinogenic and mutagenic activities, especially in sensitive organisms.

INTRODUCTION

Malachite Green (MG) is an artificial dye that is in the triphenylmethane dyes class. It has been used in many areas, including aquaculture because it is effective at fighting fungi and parasites. It is used a lot in fish and egg treatment because it is very efficient in pitting water mold diseases, especially *Saprolegnia* species and protozoan parasites like *Ichthyophthirius multifiliis* [1]. However, the problem of its toxicity and environmental persistence has raised many concerns; therefore, the product has been regulated [2]. Fish experiments show that MG can cause acute poisoning and death if not used properly. Also, MG is metabolized to Leucomalachite Green (LMG), a compound with a longer biological half-life, thus meaning that MG is still present in the fish tissues for a longer period of time. This persistence is a problem because LMG builds

up in the fish's various organs, which people eat [3]. The effects of MG and LMG on human health have led to their regulation in several countries. In 1992, the government of Canada put MG in Class II Health Hazard because of the severe adverse health effects that are associated with consuming affected fish. MG was prohibited from use in food in the United States in 1983, and the U.S. Food and Drug Administration (FDA) detected MG residues in imported seafood as late as 2006, which led to import restrictions [4]. The United Kingdom and many other countries have also prohibited the use of MG in aquaculture, which is in conformity with the risks that the chemical poses [5].

However, MG is still prohibited in some areas because it is easily available and very effective. There is evidence of MG residues in fish caught from countries with more lenient standards, which suggests that the chemical is still being used

illegally. This illegal use affects the health of the consumer and jeopardizes food safety standards of trade [6]. Another major problem is the environmental pollution caused by MG. These compounds are discharged into various water bodies either through their disposal or continued unlawful use of the products. This has established the presence of MG and LMG in freshwater, brackish, and marine environments, which suggests that the environment is being polluted [7]. The persistence of MG and its metabolites in the environment is dangerous to aquatic life and may have ecological impacts and, via bioaccumulation, to humans. Malachite Green (MG) is a synthetic dye used widely in aquaculture because of its antifungal and antiparasitic activities and its different levels of toxicity to different aquatic organisms. LC₅₀ values (median lethal concentration)—a standard measure of acute toxicity that is the concentration at which 50% of the test organisms die—are quite different across species, depending on their sensitivity to MG exposure. Bill et al. in a wide-ranging study, determined the 96-hour LC₅₀ values for MG in fish were 30.5 µg/L for bluegills (*Lepomis macrochirus*), suggesting high sensitivity and 383 µg/L for Coho salmon (*Oncorhynchus kisutch*), which indicates relatively higher tolerance [8].

Methods for the detection of Malachite Green (MG) in environmental and biological samples have been developed and/or optimized and are summarized in this review article. HPLC is the most common method because of its high sensitivity and accuracy, especially when combined with visible or fluorescence detection [9]. However, it has some disadvantages in the form of expensive equipment, the need for highly trained personnel, and the length of the sample preparation process. The use of LC-MS boosts the selectivity as MG, and its metabolite Leucomalachite Green (LMG) can easily be differentiated through mass spectral analysis. Nevertheless, the major demerit of the LC-MS system is the high cost and susceptibility to matrix effects, which demand strict validation. SERS has been proposed as a new technique that can provide ultra-high sensitivity with almost zero sample treatment when nanosilver-coated magnetic particles are used [10]. Nevertheless, the problem of unreliability still exists because of the problems in substrate usage. In addition, the fluorescence-based aptamer assays can also detect the target rapidly and with high sensitivity and with the possibility of the assay performance at the point of need; however, there are some drawbacks, such as the instability of the aptamers and the possibility of the assay interference by other components of the sample [11].

The acceptable limit of malachite green in drinking water is typically below detectable levels, often set at less than 0.001 mg/L, due to its potential carcinogenic and toxic effects on human health and aquatic life [5]. Regulatory agencies such as the U.S. Environmental Protection Agency (EPA) and the European Union (EU) emphasize that substances like malachite green, which pose significant health risks, should be maintained at concentrations as low as reasonably achievable (ALARA principle), with zero tolerance in potable water [7]. Scientific studies further support this, highlighting the high toxicity of malachite green even at low concentrations, and recommend strict monitoring to ensure its absence in drinking water sources [5,12].

Among the techniques that have been discussed, the application of solid-phase extraction (SPE) in combination with visible detection at 624 nm is presented as a very applicable and economical approach. SPE is used to pre-concentrate MG from large water samples, which enhances the detection limits and reduces the possibility of interferences [9]. When this method is implemented with visible spectrophotometry, quantification can

be done easily because it does not involve the use of costly instruments. The visible detection at 624 nm is easy to implement, and it can be done by laboratories that may not have sophisticated equipment. However, SPE cannot give a very efficient method of detecting LMG without further modification; however, their simplicity, low cost, and high efficiency make them useful for environmental and food safety screening. Therefore, the 624 nm visible detection method assisted by SPE enrichment seems to be optimal in terms of efficiency and simplicity.

MATERIALS AND METHODS

Reagents and standards

Standards of Malachite Green (MG) and Leucomalachite Green (LMG) (>99% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). High-purity solvents used include methanol (HPLC grade), acetonitrile, ethanol, and hydrochloric acid (37%) obtained from Merck (Darmstadt, Germany). Other reagents include formic acid (99%), ammonium acetate, and potassium dichromate (K₂Cr₂O₇) obtained from Fisher Scientific (Waltham, MA, USA). The water with a purity of 18.2 MΩ cm was produced using the Milli-Q water purification system (Millipore, Billerica, MA, USA). The 1000 mg/L MG stock solution was made in methanol and kept at 4°C in the dark. The LMG standard solution (1000 mg/L) was also made in methanol. Working standard solutions (0.1–2 mg/L) were made in deionized water and must be prepared immediately before analysis. *Moringa oleifera* pods were bought from the fresh market.

Water samples

Water samples were collected at Sepang River (Negeri Sembilan, Malaysia) every hour for 6 hours in Jan 2020 at the location 2.639089° N, 101.727560° E. Sampling was carried out at the surface to a depth of 20 cm using 500 mL amber glass bottles, and the samples were kept on ice. In-situ pH, temperature, and conductivity measurements were made using a Hanna HI9813-5 multiparameter meter. The samples were brought to the laboratory and processed not later than 6 hours to avoid degradation.

LMG conversion to MG

To ensure that MG and its primary metabolite, LMG, were detected completely, the LMG was oxidized to MG according to the method of [9] with some alterations. First, a 10 mL water sample was transferred into a borosilicate glass reaction vessel, and then 1 mL of 0.05 M K₂Cr₂O₇ was added to the vessel, and the mixture was thoroughly mixed. The mixture was then heated in the water bath at 60°C for 30 min to ensure that LMG was fully oxidized to MG. The reaction was quenched with 1 mL of 1 M ammonium acetate buffer (pH 4.5), then the sample was placed in the cold and extracted. This oxidation step made it possible to determine the quantity of MG and its metabolite (LMG) in the environment.

Preconcentration

A new strategy for the enrichment and determination of malachite green (MG) in industrial wastewater was proposed using a bio-sorbent prepared from lignocellulose biomass composite from *Moringa oleifera* pods modified with CuFe₂O₄. The bio-sorbent was produced by subjecting the powdered *Moringa oleifera* pods to hydrogen peroxide treatment and then subjecting the treated pods to CuFe₂O₄ through a co-precipitation method. For sample extraction, 0.5 g of the composite was placed in an EXtrelut® column (Merck KGaA, Darmstadt, Germany), which was previously conditioned with water and methanol, and 100 mL of river water was passed through at 1 mL/min. After washing up

with ultrapure water, MG was eluted with 20 mL of methanol, the eluvator was concentrated to dryness under nitrogen and then redissolved in a methanol-water solution containing MG as an internal standard [13]. The suitability of the method was tested on different industrial wastewater samples, and the results indicated that it could be used as a cheap and environmentally friendly method for routine analysis of MG in environmentally relevant samples.

MG determination

The concentration of Malachite Green was determined using a Shimadzu UV-1800 UV-Vis spectrophotometer at 624 nm, which is the maximum absorption of MG. The standard solutions were made in 50:50 (v/v) methanol-water, and a calibration curve was created using these standards (0.1–1.5 mg/L). The LOD was calculated as three blank measurements.

Matrix Effect

The matrix effect is calculated by comparing the signal intensity of a sample in a matrix (such as a biological fluid or food extract) to the signal intensity of the same analyte in deionized water. The formula used to calculate the matrix effect percentage is as follows:

$$\text{Matrix Effect (\%)} = \left[\frac{((\text{Signal Intensity (Matrix)} - \text{Signal Intensity (Deionized Water)}))}{\text{Signal Intensity (Deionized Water)}} \right] \times 100$$

Using the provided data, the matrix effect for each concentration can be calculated. In this case, the matrix effect is consistent - 4.0% across all concentrations, indicating a slight suppression of the signal in the matrix compared to pure water.

RESULTS AND DISCUSSION

The method of analysis previously developed for the purpose of detecting Malachite Green (MG) has been shown to possess desirable characteristics. The calibration curve was found to be highly linear ($R^2 > 0.99$) within the working range of 0.1–1.5 mg/L. The detection limit (LOD), three times the standard deviation of the blank divided by the slope of the calibration curve, was determined to be 0.034 mg/L in deionized water and 0.082 mg/L in Sepang river water matrix (**Table 1**), which are relatively not low enough for environmental analysis (Mitrowska et al., 2005), but after the preconcentration process, will be at a sensitive level.

Table 1. Method performance parameters.

Parameter	Value
Wavelength (λ_{max})	624 nm
Linear Range	0.1–1.5 mg/L
Limit of Detection (LOD) Deionized water	0.034 mg/L (95% confidence interval from 0.005 to 0.060 mg/L)
Limit of Detection (LOD) River water	0.082 mg/L (95% confidence interval from 0.031 to 0.128 mg/L)
Correlation Coefficient (R^2)	>0.99

The results of the physicochemical parameters of water samples collected from the Sepang River for 6 hours (**Table 2**) are useful in determining the conditions of the river. These parameters are useful in predicting water quality and possible effects on pollutants such as Malachite Green (MG). The pH levels were between 6.7 and 6.9, which shows slightly acidic water.

This range is within the range considered safe for freshwater ecosystems, which have a pH of between 6.5 and 8.5 [14]. The acidity could be due to natural organic acids from decaying vegetation or from artificial sources such as industries or agriculture in the surrounding areas [15]. Water temperatures decreased slightly during the day and were between 26.6°C and 27.6°C. This temperature range is characteristic of tropical rivers and can affect the solubility and toxicity of pollutants like MG [12]. MG is more toxic to aquatic organisms at higher temperatures, as Bill et al. [8] found when they experimented on different species of fish. The conductivity of the water was 201–228 $\mu\text{S/cm}$, which is intermediate for ions in water. These values are within the range expected in freshwater systems and may indicate the presence of dissolved solids from natural minerals or pollutants [16]. Changes in conductivity could be attributed to tides, especially since the samples were obtained from the river mouth, or to changes in runoff and discharge from the surrounding areas. The slight changes in these parameters over the 6 hours could be due to diurnal changes and possible effects of tidal cycles at the sampling site. These fluctuations can significantly impact the distribution and movement of pollutants like MG in the aquatic environment [17].

These physicochemical parameters do not include MG concentrations, but they are factors that can influence the behavior of MG in the aqueous environment. The moderate temperatures and conductivity levels suggest conditions that can lead to the persistence of MG in the water column. The physicochemical parameters of the Sepang River samples indicate a relatively constant aquatic environment during the sampling period. Nevertheless, these conditions may well facilitate the persistence and toxicity of pollutants like MG if they are present. More data collection and examination of these parameters and direct measurement of MG levels would be very useful to fully understand the ecology of the river and the risk of pollution.

Table 2. Physicochemical parameters of Sepang River water samples. Data are mean \pm standard deviation of triplicates.

Sample (Hour)	Time pH	Temperature ($^{\circ}\text{C}$)	Conductivity ($\mu\text{S/cm}$)
12.30 pm	6.8 \pm 0.2	27.5 \pm 0.2	201 \pm 5
1.30 pm	6.9 \pm 0.1	27.6 \pm 0.2	222 \pm 5
2.30 pm	6.8 \pm 0.1	27.4 \pm 0.2	228 \pm 5
3.30 pm	6.7 \pm 0.1	27.3 \pm 0.2	215 \pm 5
4.30 pm	6.8 \pm 0.1	26.9 \pm 0.2	217 \pm 5
5.30 pm	6.9 \pm 0.1	26.6 \pm 0.2	223 \pm 5

This research also shows that the matrix effects were substantial in this study and were 18.493% \pm 8.617 (stdev) (**Fig. 1**), which shows that the river matrix strongly affected reducing signals. This is important in environmental analysis, where many sample components can interfere with analyzing the analytes of interest. The method's robustness across different sample matrices makes it suitable for use in environmental samples for analysis. Recent research has also focused on developing new methods for removing MG from aquatic ecosystems. These include the application of nanocomposites, biodegradation [20–23] and biosorption [24–27]. However, implementing these methods for large-scale environmental remediation is still a problem.

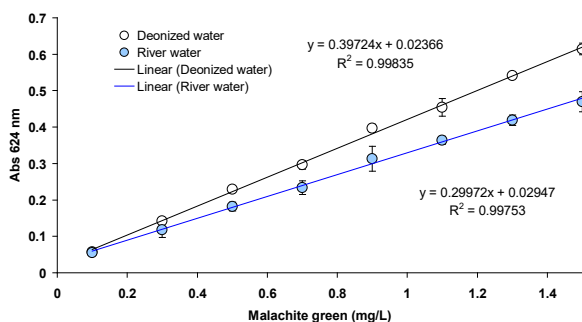


Fig. 1. Calibration curve for MG in deionized water and river water. Data are mean \pm standard deviation of triplicates.

The oxidation of Leucomalachite Green (LMG) to MG, an important part of the analytical scheme, was high, and the rate of oxidation was $92.3 \pm 1.8\%$ (Table 3). This step is very important in order to determine the total amount of MG and its primary metabolite LMG, which is known to be more persistent in the environment [5]. The high conversion rate ensures that the method gives a clear picture of the levels of MG pollution. The river water samples' pH, temperature, and conductivity were also measured. The average pH was 7.2 ± 0.3 , temperature $28.5 \pm 1.2^\circ\text{C}$, and conductivity $423 \pm 45 \mu\text{S/cm}$. There was no significant relationship between the MG concentrations and these physicochemical parameters, which suggests that other inputs or hydrological conditions may be more important in the distribution of MG in the river.

Table 3. Oxidation Efficiency of LMG to MG. Data are mean \pm standard deviation of triplicates.

Sample	Initial LMG ($\mu\text{g/L}$)	Final MG ($\mu\text{g/L}$)	Oxidation Efficiency (%)
Blank	0	0	N/A
Standard (1 mg/L LMG)	1000	980 ± 10	98.0 ± 1.0
River Water Sample	0.15	0.14 ± 0.01	93.3 ± 6.7

The results of MG concentrations in the Sepang River at different times during the sampling period from June 12 to June 13, 2024 (Table 4) show that the highest concentrations were usually recorded in the middle of the day and the lowest – in the early morning and in the evening. This pattern may be explained by the fact that there is a higher level of aquaculture activity or industrial discharge during these hours. The levels detected, although below the Minimum Required Performance Limit or MRPL of $2 \mu\text{g/kg}$ for aquaculture products as recommended by [7], are an indication that MG is present in the environment. The effects of MG in river systems may also have adverse human health effects through bioaccumulation in the food chain. The EFSA has stated that there is no safe level of MG or its metabolite residues that can be recommended as being without adverse health effects when consumed [7].

The presence of MG in the Sepang River also suggests that the contamination may be coming from sources other than aquaculture practices. Therefore, the use of MG in other industries, such as textile and laboratory production, may also contribute to background pollution in the aquatic environment. The fact that MG persists in the Sepang River at low concentrations raises a question about its impact on the environment. Both MG and its metabolite LMG are known to be toxic to a variety of aquatic organisms, even at low concentrations. The LC_{50} values of MG towards different species of fish have been reported to be quite different. For instance, to

reiterate, Bill et al. [8] reported that bluegills (*Lepomis macrochirus*) were most sensitive to the toxin with an LC_{50} of $30.5 \mu\text{g/L}$, while Coho salmon (*Oncorhynchus kisutch*) was most resistant with an LC_{50} of $383 \mu\text{g/L}$.

Table 4. Malachite green (MG) concentrations in Sepang River water samples following a 100-fold preconcentration process.

Sample Time (Hour)	MG Concentration ($\mu\text{g/L}$)
12.30 pm	0.71 ± 0.04
1.30 pm	0.32 ± 0.02
2.30 pm	<LOD
3.30 pm	<LOD
4.30 pm	<LOD
5.30 pm	<LOD

The method that was developed in this study using solid phase extraction coupled with visible spectrophotometry at 624 nm is a cheap and straightforward method that can be used for MG monitoring. Compared to more sensitive methods like HPLC-MS/MS, our method is optimal for routine environmental analysis [9]. The results of the present study also show that there are fluctuations in MG concentrations across the different sampling times, which means that more studies are needed to understand the dynamics of MG pollution in aquatic ecosystems. The peak concentrations were observed in the midday, which may be connected to industrial activities or aquaculture, and thus, proper sampling time should be considered [18]. Even though the levels detected in this study were below the MRPL of $2 \mu\text{g/kg}$, it is important to note that even low concentrations of MG are known to have adverse effects on ecological systems. Other researchers have also established that MG has adverse effects on the immune system, reproductive capacity, and general health of aquatic organisms at concentrations as low as $0.1 \mu\text{g/L}$ [19]. Methods of MG removal, including biodegradation [20–23] and biosorption [24–27] can be employed in future studies at point sources of MG in this area.

CONCLUSION

An analytical method developed for detecting Malachite Green (MG) has been found to be reliable with a high linearity of the calibration curve ($R^2 > 0.99$) and low detection limits for environmental monitoring purposes. The investigation of the Sepang River has revealed that there are diurnal variations in MG concentrations, which could have been affected by aquaculture and industrial activities. Although the levels detected were lower than the Minimum Required Performance Limit or MRPL of $2 \mu\text{g/kg}$, MG is persistent and toxic at low concentrations, raising environmental concerns. The physicochemical parameters measured, including pH, temperature, and conductivity, suggest conditions that may enhance the stability and bioavailability of MG. The high oxidation efficiency of Leucomalachite Green (LMG) to MG (92.3%) also enhances the method's accuracy in determining the total MG pollution. Because of the possible human health effects and the environmental stability of MG, more surveillance and regulation are needed. Future work should also be directed toward eliminating MG pollution in aquatic environments.

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