Sensitive Detection of Malachite Green and Crystal Violet by Nonlinear Laser Wave Mixing and Capillary Electrophoresis

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ABSTRACT

An ultrasensitive label-free antibody-free detection method for malachite green and crystal violet is presented using nonlinear laser wave-mixing spectroscopy and capillary zone electrophoresis. Wave-mixing spectroscopy provides a sensitive absorption-based detection method for trace analytes. This is accomplished by forming dynamic gratings within a sample cell, which diffracts light to create a coherent laser-like signal beam with high optical efficiency and high signal-to-noise ratio. A cubic dependence on laser power and square dependence on analyte concentration make wave mixing sensitive enough to detect molecules in their native form without the use of fluorescent labels for signal enhancement. A 532 nm laser and a 635 nm laser were used for malachite green and crystal violet sample excitation. The use of two lasers of different wavelengths allows the method to simultaneously detect both analytes. Selectivity is obtained through the capillary zone electrophoresis separation, which results in characteristic migration times. Measurement in capillary zone electrophoresis resulted in a limit of detection of 6.9×10^{-10} M (2.5×10^{-19} mol) for crystal violet and 8.3×10^{-11} M (3.0×10^{-20} mol) for malachite green at S/N of 2.

KEYWORDS

1. INTRODUCTION

As shown in Figure 1, malachite green (MG) is a triphenylmethane dye that may be used as a fungicide and paraciticide in aquaculture [1]. However, it is not approved for use as an aquaculture veterinary drug in the United States, Canada or the European Union [2-3]. Malachite green also has uses as a food coloring agent, as a medical disinfectant and as a dye for textiles [4]. Although a wide range of uses exists, it has also been reported to have toxic effects that increase with concentration, exposure time and temperature. This toxicity may present itself in the form of organ damage and mutagenic, carcinogenic and developmental abnormalities in mammals. Due to reports of this nature, the U.S. Food and Drug Administration (FDA) nominated malachite green as a priority chemical for carcinogenicity testing in 1993 [5].

As shown in Figure 1, crystal violet (CV) is another triphenylmethane dye used as an anti-microbial and anti-fungal agent for treatment of infections. It has a long history of medical use under the name Gentian Violet, but, like malachite green, crystal violet can be highly toxic and has been shown to enhance tumor growth [6-7]. Crystal violet also sees use as a DNA stain in gel electrophoresis [8], which may speak to the carcinogenicity of the compound. As is the case with malachite green, the FDA recognizes crystal violet as unsafe for use as a veterinary drug (21CFR500.29 and 21CFR500.30) [6].

Due to these health risks and the potential for illegal use in trace amounts, a very sensitive analytical detection method is needed to identify trace quantities in an aquaculture setting. Both malachite green and crystal violet are readily absorbed into fish tissues, which may then be ingested by consumers. Absorbed malachite green and crystal violet may also be metabolized to leuco malachite green (LMG) and leuco crystal violet (LCV). Current detection

methods often involve expensive, complicated techniques such as mass spectrometry to obtain low detection limits [9-15]; while many of the simpler methods are not capable of measuring below nanomolar concentration levels [16-19] This leaves a need for simple, yet sensitive, detection methods that can be field-deployed to confirm the presence of these hazards in water supplies or animal tissues. The method of detection described in this work utilizes absorptivity, molecular weight and charge to identify these drugs at sub-nanomolar concentrations by laser wave mixing coupled with capillary electrophoresis (CE). In addition to analysis of water samples, our separation and detection methods may be paired with existing sample extraction techniques [2,7,9-12,14,15,17-19] to analyze residues present in tissues.

Laser wave mixing is a powerful spectroscopic method that produces a signal through the formation of a thermal grating at the intersection point of two input laser beams (pump and pump/probe) passing through an absorbing analyte. The angle of the input beams determines the angle of the two signal beams, which allows a predictable location for collection by a photodetector. Because the input beams are derived from one laser source split by a 70:30 beam splitter, the signal beam produced from the pump/probe beam is stronger than the one produced by the pump beam. This signal has a square dependence on analyte absorptivity or concentration and a cubic dependence on laser power [20-22]. These properties allow the method to use low-power laser efficiently to excite molecules even if they do not have a large extinction coefficient. This allows a very dilute sample to produce a strong signal as described below.

(1)

In Equation 1, signal intensity is represented by I_s , and the pump beam intensities are I_1 and I_2 . b is the optical path length of the sample cell, λ_e is the excitation wavelength, θ is the angle between the pump beams, and dn/dt is the change in the refractive index with respect to

temperature. α is the absorptivity/extinction coefficient of the analyte, and κ is the solvent thermal conductivity.

Given that malachite green and crystal violet both yield strong optical absorption in the visible range, as shown in Figure 2, visible laser wavelengths closest to the peak absorption wavelengths will yield the strongest wave-mixing signals. For crystal violet, peak absorption was determined to be at 580 nm with an extinction coefficient of approximately $160,000 \text{ cm}^{-1} \text{ M}^{-1}$. Malachite green peak absorption was determined to be at 618 nm with an extinction coefficient of approximately $140,000 \text{ cm}^{-1} \text{ M}^{-1}$. The 532 nm laser excitation wavelength falls at 61% of crystal violet λ_{max} and the 635 nm laser excitation wavelength at 68% of malachite green λ_{max} . The broad absorption peak for malachite green actually allows a small amount of absorption from the 532 nm Nd:YAG laser, as well as the 635 nm diode laser used in this work. However, the same is not true for crystal violet, which does not show significant absorption near 635 nm. Furthermore, as the leuco-forms of malachite green and crystal violet (LMG and LCV) are colorless, they cannot be detected directly by the lasers used in our system. However, given the significance of these derivatives in tissue sample analysis, extensive research on sample preparation has identified oxidation reactions that convert the leuco-forms back to the original colored compounds malachite green and crystal violet [2,7,9,23]. LMG and LCV samples collected and oxidized in this manner may be analyzed concurrently with malachite green and crystal violet as aggregate peaks.

2. EXPERIMENTAL

2.1. Chemicals. Malachite green chloride and crystal violet were purchased from Sigma Aldrich. Ultratrol LN was purchased from Target Discoveries. All other chemicals were of analytical grade. Deionized water was distilled prior to use in capillary electrophoresis buffer

solutions. Buffers were filtered through 0.2 μm syringe filters prior to use as a sample diluent or in the capillary electrophoresis system.

2.2. Laser Wave Mixing System. A wave-mixing optical configuration described in our previous work [24] was utilized for analyte detection. A 200 mW 532 nm frequency-doubled Nd-YAG laser (CNI, MGL-FN-523-200mW) and a 100 mW 635nm diode laser (Laserglow Technologies, LRD-0635-TSR-00100-10) were positioned so that both beams converged at a 90-degree angle on a single 70/30 (70% transmitted, 30% reflected) beam splitter, as shown in Figure 3. This allowed approximately 70% of the 635 nm beam and 30% of the 532 nm beam to pass through the optical setup. Taking into account additional losses due to mirrors used to align the beams and spatial filters, the final effective laser powers were 65 mW and 40 mW, respectively. After the initial power reduction, the two beams were directed to a second beam splitter (70/30 R/T) to create two pairs of overlapping beams, with the weak-side beams passing through an optical chopper (SRS, SR540). These beams were then redirected by three mirrors to allow parallel paths into a 10 cm focusing lens. A polyimide coated silica capillary with 1 cm of its coating removed was placed at the focal point to allow the two pairs of beams to converge at the detection window. A spatial filter was placed behind the capillary to isolate only the strong-side signal beams from the remaining three pairs of beams. These overlapping signal beams were then reflected off of a mirror and through another 10 cm focusing lens. A photodetector (Thorlabs, PDA36A) was used to collect the signal, which was processed by a lock-in amplifier (SRS, SR810 DSP) and then digitized using our custom-built AIDA data acquisition software.

2.3. Capillary Electrophoresis System. Our custom-built capillary electrophoresis system utilized a 30 kV power supply (Glassman PS/MJ30P0400-11) with a custom-built control system to apply current across the system. Polyimide coated fused silica capillaries including 360 μm

o.d., 100 μm i.d., 35 cm (17 cm effective) and 360 μm o.d., 75 μm i.d., 40 cm (20 cm effective), were used for various experiments. Platinum electrodes were placed in glass sample vials at both end of a capillary, while system voltage and current were monitored by a multimeter and our AIDA data acquisition software. Ammonium acetate buffer (50 mM, pH 5.1) was prepared in distilled deionized water. Buffer solutions of 50 mM (pH 2.2) or 0.5 mM citric acid (pH 3.5) were prepared in distilled deionized water and then diluted 2:1 with acetonitrile similarly to methods previously reported [16,19]. MG and CV samples were prepared in 0.5 mM citric acid and acetonitrile (1:2), 50 mM citric acid and acetonitrile (1:2) or 50 mM ammonium acetate. Electrokinetic sample injection (+20 kV) was used to introduce our analytes. Prior to use, capillaries were treated with 0.1 N NaOH for 10 minutes at 5 $\mu\text{L}/\text{min}$ using a peristaltic pump, followed by priming with run buffer for 1 minute at the same flow rate. For capillary coating, Ultratrol LN capillary dynamic coating polymer (Target Discoveries) was flowed into the capillary at 5 $\mu\text{L}/\text{min}$ between the standard 0.1 N NaOH and run buffer treatment steps. Between sample runs, capillaries were flushed with 0.1 N NaOH and run buffer for 1 minute at 5 $\mu\text{L}/\text{min}$.

3. RESULTS AND DISCUSSION

3.1. Background Electrolytes. Initial attempts to detect MG and CV in the capillary electrophoresis mode were made using an aqueous 50 mM ammonium acetate buffer (pH 5.1). This buffer was chosen due to its acidic pH and the successful application of a similar solution [19]. However, when used as the sample solvent and background electrolyte in the capillary electrophoresis system, the current was found to be excessively high ($> 240 \mu\text{A}$) at 20 kV applied voltage. Through multiple sample injections with this buffer, only two successful runs were recorded without loss of system conductivity. Despite the use of long injection times, signal response was found to be relatively poor in the 50 mM ammonium acetate system. The weak

signal, combined with the frequent boiling of the buffer due to high current, ultimately led to a change in CE run buffer.

While a reduction in ammonium acetate concentration would have reduced system current to more manageable levels, the signal would have still been weak and the sensitivity for MG and CV detection would have been limited. For this reason, another acidic run buffer was formulated using 50 mM citric acid. This system was chosen for its reported success as a capillary electrophoresis buffer in a 2007 work [16]. Initial testing of this 50 mM citric acid solution in the CE system showed a significant reduction in current to an average of 70 μA with a constant +20 kV applied voltage. This current decrease vs. ammonium acetate is due to a change in conductivity resulting from the higher molecular weight of citric acid. Although a more stable CE system was possible with this buffer, the wave-mixing signal response was found to be even lower than with the ammonium acetate buffer. The electropherogram peaks were also very broad and non-Gaussian in shape, as shown in Figure 4A. This observation suggested poor analyte solubility in a purely aqueous citric acid solution, which may have resulted in capillary wall interaction. In light of this result, the decision to add a significant organic component to the run buffer was made. The 50 mM aqueous citric acid solution was then diluted 1:2 with acetonitrile to produce a more suitable run buffer. When this new buffer was used in a CE separation run, the reduction in net citric acid concentration and the change in the dielectric constant were found to further reduce the average system current to 10 μA at +20 kV. This allowed for highly consistent electropherograms and minimal instances of total current loss. The change also resulted in nearly a 50-fold increase in the wave-mixing signal, as shown in Figure 4B, when compared to that of 50 mM citric acid alone (Figure 4A). This dramatic increase is due to a combination of effects. First, CV and MG both have greater absorptivity in acetonitrile than

in water. Second, solubility of both compounds is increased in acetonitrile, which may have reduced capillary wall interactions and improved electrokinetic injections. Given the observations made with each of these capillary electrophoresis run buffers, the 50 mM citric acid: acetonitrile (1:2) solution was the clear choice in terms of signal response and system stability. This run buffer was used for all further capillary electrophoresis runs.

3.2. Field Amplified Sample Stacking. Sample preparation can have a major impact on separation and detection of analytes in capillary electrophoresis. Sample stacking is a preparation technique that aims at pre-concentration of analytes prior to separation to increase efficiency. There are numerous types of sample stacking procedures; however, many of these methods require complex control over the capillary system. Field amplified sample stacking (FASS) is a simple stacking method that can yield large increases in signal-to-noise ratio (S/N) with only minor system modifications [25]. By injecting samples in a low-conductivity solvent, analytes experience a strong electric field, and therefore, migrate at an increased rate relative to those injected in higher conductivity solvents. Analytes in a low-conductivity zone slow once the interface with a high-conductivity run buffer is reached, which causes a stacking effect. Due to the limited injection capability of our capillary electrophoresis system, this technique was chosen for separation of MG and CV.

Figure 5 shows electropherograms for 10 μ M CV prepared in both 50 mM citric acid/acetonitrile (1:2) and 0.5 mM citric acid/acetonitrile (1:2). The 50 mM citric acid/acetonitrile (1:2) was used as run buffer for both runs. The peaks obtained showed a dramatic difference in S/N, with the sample prepared in low-conductivity stacking buffer yielding nearly a 5-fold increase over the sample prepared in run buffer. Given this significant

improvement in detection sensitivity, all further samples were prepared in 0.5 mM citric acid/acetonitrile (1:2) stacking buffer.

3.3. Bare Capillary Separation. Figure 6A shows a typical electropherogram for a mixture of 10 μ M MG and 10 μ M CV using capillary zone electrophoresis (CZE). Eleven successive injections of the sample mixture were performed to observe variation in run-to-run migration times. Migration times for each compound showed good repeatability at 57-65 seconds (RSD of 5.2%) for MG and 60-71 seconds (RSD of 5.6%) for CV, as shown in Figure 6B, despite a lack of temperature control or automated sample injection. For all trials, both MG and CV peaks were resolved from one another; however, it was observed that after 3 to 4 runs, migration times for both compounds increased. This is most evident with the migration times for runs 3 and 8, which show a significant increase in relation to the previous run. Additional treatment of the capillary with 0.1 N NaOH was performed for 1 minute after each of these runs, and migration times were found to stabilize. This change in migration times is the result of re-protonation of capillary silanol groups due to the use of an acidic run buffer. Decreases in the net negative charge on the capillary surface reduce electroosmotic flow (EOF), while treatment with base serves to restore the net negative charge and EOF. Further reduction in run-to-run RSD may be obtained through flushing the capillary with 0.1 N NaOH between runs.

Another observation from this experiment is the difference in analyte wave-mixing signal strengths. The signal for MG is significantly stronger than that of CV due to two factors. First, the 635 nm laser has a greater effective power (65 mW) than that of the 532 nm laser (40 mW). Since wave-mixing signals have a cubic dependence on laser power, a higher intensity signal is generated for MG. Additionally, MG also absorbs at 532 nm allowing for signal formation from

both input lasers. This amounts to total MG signal strength that is approximately 5 times greater than that generated by CV. The peaks shown in Figure 6A reflect this difference.

3.4. Effects of Capillary Dynamic Coating. Although separation of MG and CV was successfully obtained using a bare fused-silica capillary, a degree of system control is required to prevent shifts in migration time from causing peak overlap between runs. To this end, modification of the CZE system could potentially allow for improved separation. One means of creating greater separation between analytes is through reduction of electroosmotic flow. Dynamic capillary coatings offer a simple means of EOF reduction through non-permanent alteration of the capillary surface.

Figure 7 shows CE separation of MG and CV in a 100 μm i.d. capillary with and without Ultratrol LN dynamic coating. In the uncoated capillary, MG and CV were detected at 66.3 and 72.3 s. However, the addition of Ultratrol LN shifted these migration times to 90.6 and 98.4 s, respectively. This is a 30% improvement in migration time differential; however, there was no net gain in overall peak resolution with this modification. In both cases, band broadening negatively affected peak shape, which negated improvement in migration time change. For this reason, bare capillaries were used for further measurement of samples.

3.5. Detection Limits. To determine the lowest detectable concentration of both MG and CV, stock solutions were prepared at high concentration (1.0×10^{-3} M) in 0.5 mM aqueous citric acid/acetonitrile (1:2) stacking buffer. Serial dilutions were then made ranging from 1.0×10^{-4} M to 1.0×10^{-12} M and tested individually. It should be noted that after initial optical alignment with high concentration samples, no changes were made to the positions of mirrors, spatial filters or the sample cell. The only changes made during this process were to detector gain and signal amplification scaling. Figure 8 shows the lowest concentration MG and CV samples (2.0×10^{-10}

M MG and 2.0×10^{-9} M CV) that generated a detectable wave-mixing signal. From these electropherograms, the limits of detection were calculated using peak height (signal) and the standard deviation of the baseline (noise). Preliminary concentration detection limits of 8.3×10^{-11} M for MG and 6.9×10^{-10} M for CV (S/N=2) were determined. Using an estimated laser probe volume of 360 pL, preliminary mass detection limits of 3.0×10^{-20} mol for MG and 2.5×10^{-19} mol for CV were determined. The sample size detected is very small, since wave mixing only requires a micrometer thin optical path length and the focused laser beam diameter is smaller 70 μm .

Investigation of the technique as a quantitative tool led to generation of standard curves using a series of MG and CV calibrators. For each set of calibrators, instrument response vs. analyte concentration was characterized. Instead of electrokinetic injection, each sample was drawn into a 75 μm i.d., 360 μm o.d., 40 cm capillary using a peristaltic pump at a continuous flow rate of 5 $\mu\text{L}/\text{min}$. Wave-mixing signal produced by each sample was monitored over a 30 second period (10 data points/s). This allowed for direct evaluation of wave-mixing signal linearity without variation introduced by the capillary electrophoresis system. Future method development using commercially-available capillary electrophoresis instruments coupled with our wave-mixing detector is expected to allow for traditional linearity assessment under controlled electrophoretic conditions.

The mean signal values obtained for 1.0 – 50 μM CV samples are shown in Figure 9A. Given the square dependence of wave-mixing signal on absorptivity, a second-order polynomial response was observed. On a logarithmic scale, a slope of approximately 2 was observed as expected (Figure 9B). Linear fit over this range was found to be good, with the relationship defined as $y = 1.784x + 8.045$ ($R^2 = 0.993$). Similarly, Figure 9C and Figure 9D illustrate mean

wave-mixing signals for 6.25 - 50 μ M MG calibrator samples on both absolute and logarithmic scales. Linear line fit for these samples also resulted in a high correlation coefficient ($y = 1.736x + 7.615$, $R^2 = 0.996$).

4. CONCLUSIONS

Laser wave mixing coupled with capillary electrophoresis provides a highly sensitive detection method for malachite green and crystal violet. The method does not require any derivatization or labeling of the target analytes, and it is capable of producing reproducible results in less than two minutes. It yields picomolar detection limits in a detection system that can be scaled down to field-deployable designs, while other published techniques do not offer both sensitivity and portability, as shown in Table 1 and Table 2. Further development of this method may offer even greater sensitivity and reproducibility through more advanced sample stacking techniques and enhanced system controls.

The objective of the work to demonstrate a highly sensitive test method for simultaneous detection of malachite green and crystal violet has been achieved. The method is compatible with existing extraction techniques provided filtration and dialysis into the appropriate sample buffers, and it may also be used to detect the metabolites leuco malachite green and leuco crystal violet if samples are oxidized using published sample preparation methods.

Malachite Green

MW = 329.458 g/mol

Crystal Violet

MW = 370.526 g/mol

Figure 1. Structures of malachite green and crystal violet.

Figure 2. UV-visible spectra of malachite green ($1.0\ \mu\text{M}$) and crystal violet ($1.0\ \mu\text{M}$).

Figure 3. Experimental setup for wave-mixing detection of malachite green and crystal violet. 532 nm and 635 nm lasers are used for simultaneous detection. S1, S2: Beam splitters; B1, B2: Beam blockers; L1, L2: Lenses.

Figure 4. 1×10^{-4} M crystal violet (CV) in 50 mM citric acid (A). 1×10^{-4} M CV in 50 mM citric acid diluted with acetonitrile (1:2) (B). Capillary electrophoresis parameters: 20 kV, 75 μm i.d., 360 μm o.d., 40 cm capillary (20 cm effective length), 30 s (A) and 5 s (B) electrokinetic injections.

Figure 5. Field Amplified Sample Stacking (FASS) electropherograms. 10 μ M CV in 50 mM aqueous citric acid/acetonitrile (1:2) (Left). 10 μ M CV in 0.5 mM aqueous citric acid / acetonitrile (1:2) stacking buffer (Right). Capillary electrophoresis parameters: 20 kV, 75 μ m i.d., 360 μ m o.d., 40 cm capillary (20 cm effective length); 5 s electrokinetic injection.

Figure 6. (A) Electropherogram of a mixture of 10 μM MG and 10 μM CV in 0.5 mM aqueous citric acid/acetonitrile (1:2) stacking buffer. (B) Migration times for repeated injections of the same sample mixture in (A); MG (○), CV (◇). Capillary electrophoresis parameters: 20 kV, 100 μm i.d., 360 μm o.d., 35 cm capillary (17 cm effective length), 10 s electrokinetic injection.

Figure 7. Electropherograms of a mixture of 10 μM MG and 10 μM CV in 0.5 mM aqueous citric acid/acetonitrile (1:2) stacking buffer. (Top) Bare fused silica capillary, (Bottom) Ultratrol LN dynamic capillary coating. Capillary electrophoresis parameters: 20 kV, 100 μm i.d., 360 μm i.d., 35 cm capillary (17 cm effective length), 3 s electrokinetic injection.

Figure 8. Electropherograms of $2.0 \times 10^{-10} \text{ M MG}$ and $2.0 \times 10^{-9} \text{ M CV}$ in 0.5 mM aqueous citric acid/acetonitrile (1:2) stacking buffer. Capillary electrophoresis parameters: 20 kV, 100 μm i.d., 360 μm o.d., 35 cm capillary (17 cm effective length); 15 s electrokinetic injection.

Figure 9. (A) Signal vs. concentration for CV. (B) Log(signal) vs. log(concentration) for CV. (C) Signal vs. concentration for MG. (D) Log(signal) vs. log(concentration) for MG. 75 μm i.d., 360 μm o.d., 40 cm capillary.

Table 1. Detection limits for crystal violet detection methods.

Method	Detection Limit	Reference
LC–QqQLIT-MS/MS	3.29×10^{-11} M (12.2 ppt)	[11]
LC-ESI-MS/MS	4.1×10^{-10} M (152 ppt)	[12]
CE-D4WM	6.9×10^{-10} M (256 ppt)	This Work
ELISA	1.01×10^{-9} M (374 ppt)	[14]
LC–MS/MS	2.2×10^{-9} M (815 ppt)	[10]
SPE-UV/Vis	1.23×10^{-9} M (456 ppt)	[7]
CE - UV/Vis	9.8×10^{-8} M (36.3 ppb)	[17]

Table 2. Detection limits for malachite green detection methods.

Method	Detection Limit	Reference
TiO ₂ SERS Sensor	1×10^{-12} M (0.3 ppt)	[13]
LC–QqQLIT-MS/MS	3.29×10^{-11} M (10.8 ppt)	[11]
CE-D4WM	8.3×10^{-11} M (27.3 ppt)	This Work
LC-DAD	1.08×10^{-10} M (35.6 ppt)	[18]
LC-ESI-MS/MS	4.1×10^{-10} M (135 ppt)	[12]
ELISA	6.33×10^{-10} M (209 ppt)	[15]
LC-IT-MS	6.85×10^{-10} M (226 ppt)	[9]

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