Research Article

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A proposed image-based detection of methamidophos pesticide using peroxyoxalate chemiluminescence system

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Abstract: Pesticides pose a serious public health risk due to their toxicity, such as in the case of the widely distributed organophosphorus pesticide methamidophos. There is a strong need to develop a simple, rapid, and cost-effective method of detecting methamidophos residues; thus, this study proposes the TCPO-Rubrene- H_2O_2 chemiluminescence (CL) system as a means of pesticide detection via quenching effect. The results show that the methamidophos concentration is inversely proportional to the CL system's light output as confirmed through fluorescence spectroscopy and Batch Measure Macro (BMM) analysis. The light intensity differences were correlated with the methamidophos concentration with both methods showing linear trends. Both the digital camera and the smartphone camera BMM analyses displayed good sensitivity, with respective detection limits of 1.6 $\mu\text{g/mL}$ and 1.0 $\mu\text{g/mL}$ and respective quantitation limits of 5.0 $\mu\text{g/mL}$ and 3.0 $\mu\text{g/mL}$. Both also showed good linearity within the 100–10000 $\mu\text{g/mL}$ range, suggesting viability as alternatives to the fluorescence spectrometer; however, the light intensity difference values per pesticide concentration of both camera systems were significantly different from one another owing to differences in camera features.

Keywords: rubrene; smartphone camera; chemiluminescence; Batch Measure Macro; image analysis.

1 Introduction

The mishandling of pesticides in different agricultural and household applications can lead to the contamination of soil and local food produce, posing a major health and food safety concern due to the inherent toxicity of most pesticide components. As a result, this issue has initiated many pesticide detection studies which are used to either confirm the contamination of food or an area, or act as a preventive measure to avoid pesticide contamination and thereby upholding public health [1]. Many pesticide detection studies are focused on the detection of organophosphates, a class of compounds that are considered as the most widely used insecticides worldwide and have a high level of distribution in the Philippines. Their widespread use make them a good target compound of interest for future detection studies [2]. In particular, methamidophos is one specific type of organophosphorus pesticide that has been highlighted as the most frequently used organophosphorus pesticide in the Philippines during the years 2004 to 2009 and posed significant health risks to Filipino farmers [3].

Chemiluminescence (CL) serves as a good technique in the detection of these pesticide residues, where its qualities of high sensitivity and ease of use prove to be advantageous in detecting various amounts of chemical species, such as organophosphates, at relatively small amounts [4]. Moreover, CL is considered as a more cost-efficient method than other detection systems, such as gas chromatography and high pressure liquid chromatography [5]. Specifically, the peroxyoxalate CL system (PO-CL) is a well-established analytical CL scheme that is considered as one of the most efficient non-enzymatic chemiluminescent reactions known [6]. It is generally explained through the proposed Chemically Initiated Electron Exchange Luminescence (CIEEL) mechanism [7,8] that involves hydrogen peroxide oxidation of a peroxyoxalate derivative, such as bis(2,4,6-trichlorophenyl) oxalate (TCPO), which produces an excited intermediate postulated as 1,2-dioxetanedione [4,7]. More recent experimental evidence to support

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Figure 1: Schematic diagram (left) and top view (right) of the wooden lightproof boxes used in the study's analyses.

this hypothesized high-energy intermediate in a PO-CL reaction have been reported [5]. The unstable compound forms a charge complex with a fluorophore [10,11], thus raising the fluorophore to an excited state and subsequently liberating its light characteristic [8].

In choosing from the wide variety of fluorophores for a good candidate to use in pesticide CL detection studies, polycyclic aromatic hydrocarbons (PAHs) appear to be the best acceptors [6]. In particular, rubrene (5,6,11,12-tetraphenylnaphthacene), a red colored PAH, has been highlighted in previous studies as a good component in chemiluminescent activities, releasing an orange to yellow light through the CIEEL mechanism [8]. Highly oxidizable compounds such as pesticides are known to affect the luminosity of various CL systems by quenching the light intensity output [6,7]. Previous studies have already established the ability of different chemiluminescent techniques in detecting organophosphate pesticides such as the peroxyoxalate-3-aminofluorathane system [9] and luminol- H_2O_2 [10]. It was thus hypothesized that the reaction of methamidophos with the TCPO-Rubrene- H_2O_2 system can be used as basis for the detection. Compared to the other chemiluminescent detection systems for organophosphates that mainly rely on HPLC, this study's proposed detection system presents numerous advantages such as relatively simple, rapid and inexpensive instrumentation process; high portability; and relatively low reagent consumption.

The main purpose of the study is to investigate whether organophosphorus pesticide methamidophos residues could be detected through the CL reaction of

the TCPO-Rubrene- H_2O_2 system. It also aims to examine the change in the luminosity of the PO-CL system when mixed with the target compound methamidophos and to elucidate the effect of methamidophos concentration on the light intensity output of the system by applying the proposed method to spiked water samples. Moreover, the research aims to present the Batch Measure Macro (BMM) analysis as a fast, sensitive and cost-efficient method and as an alternative to the heavy equipment used in measuring light intensity output of a CL system, such as fluorescence spectrometer. Lastly, it aims to check if there is a significant difference between the readings of the digital camera and the smartphone camera in the BMM analysis.

2 Experimental

2.1 Materials and methods

All reagents used in the study were of analytical reagent grade. TCPO (Bis(2,4,6-trichlorophenyl) oxalate), rubrene (5,6,11,12-tetraphenylnaphthacene), hydrogen peroxide (H_2O_2), were purchased from Sigma-Aldrich Singapore. Ethyl acetate was purchased from Pharmco-Aaper. The materials utilized in the BMM analyses include clear vials of 10 mL capacity, and two fabricated wooden lightproof boxes sized 15.2 x 12.7 x 12.7 cm, with a hole of 7.6 cm diameter on one side to allow the lens of the cameras to be inserted and to be focused on the vials (see Figure 1).

The camera systems used for the BMM analyses include a digital camera (Nikon D5100 DSLR) and a smartphone camera (Apple iPhone 7 Plus). Spectroscopy analysis was done at the University of Santo Tomas Analytical Services Laboratory using a PerkinElmer LS 45 Fluorescence Spectrometer. Software tools used in the study include ImageJ to analyze the image samples, and GraphPad Prism 7 to conduct statistical analyses.

2.2 Solution Preparation

Luminescing solutions were prepared using a published methodology [6]. The fluorescing solutions contained two components, named as Solution I and Solution II. Solution I was comprised of TCPO (0.01 M in ethyl acetate) and the fluorophore rubrene (0.01M in ethyl acetate) in a 1:2 ratio while Solution II contained H₂O₂ (30%) and sodium acetate (0.1) in methanol in a 1:2 ratio.

A standard solution of methamidophos pesticide was prepared by dissolving it in 100 μ L methanol, following the methodology described elsewhere [4]. This solution was diluted with distilled water to achieve the required concentrations for testing; 1, 10, 100, 1000, 5000, and 10000 μ g/mL. For the negative control, 100 μ L distilled water was used.

2.3 Change in light intensity output determination through fluorescence spectroscopy

The resulting mixture of Solution I and Solution II were subsequently mixed with water samples spiked with pesticide. The CL readings were recorded before and immediately after mixing the solutions. Quenching in the luminosity was checked to verify any reactions that occurred. Three trials for each amount were conducted to ensure consistency and accuracy and for statistical analysis. Since the emission profile of CL is similar to that of fluorescence, light intensity outputs were read through the fluorescence spectrometer.

A blank solution, which contained 80 μ L of dilute Solution II and 10 μ L of Solution I was first tested. Subsequent readings were then performed by adding 10 μ L of the different concentrations of pesticide samples, along with the negative control. The results were plotted as fluorescence curves where the maximum peaks served as the CL signals per pesticide concentration. The obtained CL signals were used to calculate the CL intensity difference (ΔI) values per pesticide concentration using

Eq. 1, wherein I_x and I_o are the CL signals in presence and absence of the pesticide, respectively.

$$\Delta I = I_o - I_x \quad (1)$$

The calculated CL intensity difference values were correlated with their respective pesticide concentration. The determination method was based on the relationship between ΔI and the corresponding concentration of pesticide.

2.4 BMM Analyses

In this method, 1.5 mL Solution I were mixed with 3 mL Solution II in clear vials to produce the luminescing effect. These vials were placed inside the lightproof boxes, on a platform with a fixed distance of 7.6 cm from the camera lens and then photographed. Afterwards, the luminescing solutions were added to 100 μ L water samples spiked with methamidophos to produce the quenched solutions. Similar steps were taken to acquire pictures of the quenched solution. Capturing of photos was done in triplicates.

To obtain the CL signals, the pictures of the luminescing and quenched solutions were transferred to ImageJ software and were analyzed using the background subtraction method of the software. The resulting CL signals were used to compute the CL intensity difference values using Eq. 1.

To ensure that no external light source is contributing to the light intensity output of the luminescing and quenched solutions, ImageJ analysis via the HiLo filter was applied to a test photo taken inside the empty box.

2.5 Statistical analyses

Scatter plots showing the relationship between the light intensity difference and the pesticide sample concentration were prepared based on the normalized light intensity. To determine the effect of the methamidophos samples on the luminescent activity of the CL system the CL readings, acquired from both the fluorescence spectroscopy and the BMM analyses, among concentrations were analyzed using One-Way Analysis of Variance (ANOVA) with Tukey's multiple comparisons through the GraphPad Prism 7 software. This determined if the light difference readings were significantly different from one another. Moreover, to check for any significant differences between the readings of the digital camera and the smartphone

Figure 2: Generally accepted understanding of the quenching mechanism of PO-CL in the presence of quencher [6].

camera, the acquired slopes of each graph were compared using unpaired t-test.

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and Discussion

3.1 Chemistry

The fluorophore rubrene in its excited state produces a yellow glow of light with emission wavelength of 540 nm. The proposed mechanism for the luminescence of rubrene involves the formation of a high energy intermediate 1,2-dioxetanedione, from the reaction of TCPO and H_2O_2 , which then produces a charge complex with the fluorophore rubrene, the former donating an electron to the latter. The transfer of electron raises rubrene to an excited state [8] thus liberating its light characteristic of an orange to yellow light [6].

Peroxyoxalate CL can be affected by addition of easily oxidizable compounds like sulfate, nitrates, and

organosulfur compounds which can serve as quenchers in solution [12]. Quenchers when reacted to C_2O_4 yield non-chemiluminescent products, in competition with rubrene [7]. This quenching mechanism of the TCPO-Rubrene- H_2O_2 CL by methamidophos, a highly oxidizable compound, could be modeled as an electron transfer quenching pathway. This pathway postulates that as rubrene gets reduced, the resulting rubrene anion may react with C_2O_4 to produce $\text{C}_2\text{O}_4^{\cdot-}$. This results in a diminished amount of the reactive intermediate in solution; consequently, this would result to a reduced intensity of the CL output. The pesticide, acting as the quencher, may also undergo a reduction reaction with the excited state rubrene, resulting in a ground state of rubrene without the release of light energy, thus further reducing the CL output of the system (Figure 2) [6].

3.2 Fluorescence Spectroscopy

The obtained fluorescence spectroscopy results were averaged per wavelength number, then the averaged data were used to generate the fluorescence spectra shown in

Figure 3. Figure 3 shows five graphs with varying light intensity peaks at different wavelengths as summarized in Table 1. It was verified through One-way ANOVA analysis with Tukey's multiple comparisons that all light intensity peaks of every concentration for both light intensity peaks were significantly different at the 95% confidence interval, indicating the high sensitivity of the CL system to changes in pesticide concentration.

From the spectra, it can be seen that light intensity peak decreases as the pesticide concentration increases, as the blank solution (0 $\mu\text{g/mL}$) displayed the highest light intensity peak while the solution with the highest pesticide concentration (10000 $\mu\text{g/mL}$) showed the lowest light intensity peak (See Figure 3 and Table 1). A similar inverse trend was observed in the previous studies of Ohtomo et al. [13] in determining trace amounts of L-tyrosine contained in dietary supplements, and Khajvand et al. [6] in studying the effects of glutathione, L-cysteine, and L-methionine on the kinetics and mechanism of the peroxyoxalate–rubrene– H_2O_2 chemiluminescence system. Moreover, quenching effects and light intensity-pesticide concentration inverse relationships were also found in other CL pesticide detection studies, such as in the case of diazinon, malathion and phorate in a rhodamine B-covered gold nanoparticle-based (RB-AuNP) assay [14].

In addition, since an electron transfer quenching pathway can be modelled for the possible quenching mechanism of the pesticide, the concentration of quenchers can affect the light intensity output. If the pesticide concentration is higher in the solution, there will be a higher concentration of quenchers which may undergo reduction and bind with the high energy intermediate C_2O_4 or excited state rubrene to produce non-chemiluminescent products; therefore, the amount of light intensity output is more diminished [7,12]. Similarly, the lower the pesticide concentration, the higher the light intensity output. Also, according to the study of Khajvand, et al. which studied the effect of L-methionine –which has a similar $-\text{S}-\text{CH}_3$ substituent to methamidophos– on the same CL system, another possible mechanism of quenching is due to the heavy atom effect. The sulfur atom of the methamidophos leads to the intersystem crossing (ISC) via the singlet excited state of rubrene to the appropriate triplet excited state. This ISC mechanism resulted in the reduction in the singlet excited state concentration of rubrene and also in the reduction of the light intensity output [6].

The slight differences in the wavelength maxima of the light intensity peaks suggest possible reabsorption of the shorter wavelength part of the emission, a phenomenon that occurs with small changes in concentration brought by the large Stokes shift of rubrene [15,16]. However, as

Figure 3: Fluorescence spectra of the blank solution (red) and quenched solutions with varying pesticide concentrations: 10000 (blue), 7500 (yellow), 5000 (grey), 1000 $\mu\text{g/mL}$ (orange), and 0 $\mu\text{g/mL}$ (red).

Table 1: Summary of the mean light intensity peaks with their wavelengths of all pesticide concentrations (Fluorescence spectrometer).

Pesticide Concentration ($\mu\text{g/mL}$)	Mean Light Intensity Peaks (a.u.) ($\pm$ s.d.)	Wavelength (nm)
0	516.841 ± 0.353	551
1000	498.127 ± 1.328	537
5000	441.051 ± 0.892	546.5
7500	397.810 ± 0.877	552.5
10000	373.547 ± 1.206	544

seen in Table 1, the light intensity peaks of all samples were located at different wavelength in the range of 537–551 nm, which is still part of the wavelength interval for the yellow light characteristic of rubrene [17]. Therefore, the differences in the wavelength do not significantly affect the system since the system relies on changes in the light intensities.

The relationship between the ΔI and pesticide concentration was also investigated in this fluorescence spectroscopy analysis, and it resulted in a direct correlation (see Table 2). As the pesticide concentration increases, the light intensity difference increases. This is correct given that the light intensity peak and pesticide concentration are shown to be inversely proportional. With a higher concentration of pesticide, a lower light intensity peak is produced; thus, the difference with the light intensity of the blank solution is higher.

The light intensity peak was changed to light intensity difference, as it is important that the determination of pesticide concentration by quenched CL is based on the decrease in the resulting signal. This is to reduce the noise present in the luminescence detection as much as

possible and to achieve a favorable signal to noise ratio for quenched CL [6]. The graph for mean light intensity difference values and pesticide concentration showed good linearity, showing the possibility of using the CL system to detect the pesticide concentration on a given range.

3.3 BMM Analyses

ImageJ software has been utilized in previous chemiluminescence imaging studies suggesting its potential for analyzing chemiluminescent data [18]. Prior to sample analysis, the lightproof box was examined to test for the effect of external light sources and interference in the measurements using the method for Background Correction indicated in the ImageJ website (http://imagej.net/Image_Intensity_Processing). The HiLo filter displays zero values of light output as blue, and thus the predominantly blue colored photo obtained from the HiLo filtered image of the empty lightproof box indicates that there was almost zero external light contributing when photos are captured. With the near-zero external light source, noises from the background were reduced as much as possible in order not to interfere with the actual light intensity readings from the luminescing and quenching solutions.

Background subtraction through the ImageJ software was employed for both the digital camera and smartphone camera BMM analyses in obtaining the total light reading from each solution. This was done to obtain more accurate light intensity readings and to reduce as much as possible the noise from the background of the photo. Average ΔI was also used in BMM analyses in correlating pesticide concentration and light intensity output.

When different concentrations of pesticide spiked water samples were tested in the luminescing solutions, both of the BMM analyses resulted in a direct linear correlation between the pesticide concentration and the light intensity difference. As the pesticide concentration decreases, the light intensity output increases (see Figure 4). The quenched solution with the highest pesticide concentration (10000 $\mu\text{g/mL}$) displayed the lowest light intensity output, while the quenched solution with the lowest concentration (1 $\mu\text{g/mL}$) showed the highest light intensity output. These findings correlate with the findings from the fluorescence spectroscopy analysis, which then further verifies the inverse relationship of the pesticide concentration and light intensity.

The mean difference in the light intensity outputs of the luminescing and quenched solutions of every setup

Table 2: Summary of the mean light intensity difference of all pesticide concentrations (Fluorescence spectrometer).

Pesticide Concentration ($\mu\text{g/mL}$)	Mean Light Intensity Difference (a.u.) ($\pm$ s.d.)
0	0 ± 0
1000	18.713 ± 0.976
5000	75.789 ± 1.020
7500	119.031 ± 0.525
10000	143.293 ± 1.195

for the digital camera BMM and the smartphone camera BMM analyses are shown in Figure 5. The data points in each analysis were all significantly different at the 95% confidence level using One-way ANOVA with Tukey's multiple comparisons, which denotes that the CL system has high sensitivity.

3.4 Statistical Analysis

The graphs of the two BMM analyses contained two segments with different slopes at the same pesticide concentration intervals, which are 1-100 $\mu\text{g/mL}$ and 100-10000 $\mu\text{g/mL}$. The slopes for the two segments were found to be significantly different using an unpaired t-test at the 95% confidence interval, which means that there is a particular linear equation for each pesticide concentration interval. A possible factor for this phenomenon is the automatic setting adjustment of the cameras under different light conditions. Under high concentrations of the pesticide, the changes in light output and in the quenching of the chemiluminescence can be readily observed by the naked eye and result in a dark environment for the picture; on the other hand, the opposite is true for the low concentrations of pesticide and results in an environment with sufficient light [19]. Since the cameras automatically adjust exposure under dark conditions, the camera settings may have been different for the high concentrations and low concentrations, resulting into two different readings and subsequently resulting into two different slopes.

From the graphs in Figure 5, four different linear equations were obtained. The following equations were written as follows: (1) 1-100 $\mu\text{g/mL}$ and (2) 100-10000 $\mu\text{g/mL}$ intervals for the digital camera BMM Analysis, and (3) 1-100 $\mu\text{g/mL}$ and (4) 100-10000 $\mu\text{g/mL}$ intervals for the smartphone camera BMM Analysis.

$$y = 175.12x + 410639 \quad (2)$$

Figure 4: The resulting quenched solutions in the vials when the water samples with pesticide concentrations 10000, 5000, 1000, 100, 10, and 1 $\mu\text{g/mL}$ respectively were added to their luminescing solutions. The top images were taken using the digital camera while the bottom images were taken using the smartphone camera.

Figure 5: Graph of the light intensity difference and pesticide concentration (1-10000 $\mu\text{g/mL}$) for the digital camera BMM Analysis (left) and the smartphone camera BMM Analysis (right).

$$y = 3076.9x + 59945$$

$$y = 280.58x + 1460402$$

$$y = 12729x + 84773$$

- (3) 5 for the smartphone camera) illustrated good linearity, with R^2 values of 0.9927 and 0.9922, respectively. On the other hand, for the two equations for the 1-100 $\mu\text{g/mL}$ concentration interval (Eq. 2 for the digital camera and Eq. 4 for the smartphone camera) only Eq. 4 showed good linearity, with a R^2 value of 0.9915, while Eq. 2 exhibited a relatively low R^2 value of 0.8856. These results suggest that at higher concentration values of 100-10000 $\mu\text{g/mL}$,

Both of the equations for the 100-10000 $\mu\text{g/mL}$ pesticide concentration interval (Eq. 3 for the digital camera and Eq.

BMM analyses using either camera setups may be used as alternatives to the fluorescence spectrometer and still yield optimal readings. Meanwhile, the findings also suggest that only the smartphone may possibly be applied as an alternative to fluorescence spectroscopy at lower concentration values of 1-100 µg/mL.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated by employing the calibration curve slope method where the acquired slopes of Eq. 2 and Eq. 4 were inputted to Eq. 6 [20].

$$\text{LOD/LOQ} = (F \times \text{SD})/b \quad (6)$$

where F is a factor of 3.3 and 10 for LOD and LOQ, respectively; SD is the standard deviation of the blank (84.4775 for the digital camera BMM and 87.1425 for the smartphone camera BMM); b is the slope of the regression line (280.58 for the digital camera BMM and 175.12 for the smartphone camera BMM). The values used in this evaluation were derived from the regression model presented in Figure 5. The computed LOD and LOQ for the BMM analysis using the smartphone were 1.0 µg/mL and 3.0 µg/mL, respectively. Meanwhile, the LOD and LOQ for the BMM analysis using the digital camera were extrapolated to be 1.6 µg/mL and 5.0 µg/mL, respectively. It is important to quantify the LOD and LOQ of the BMM analyses in order to validate the capability of the method for detecting methamidophos at lethal concentrations. Methamidophos is known to be poisonous to aquatic organisms, with a lethal concentration 50 (LC_{50}) in water reported to be 25-51 µg/mL in rainbow trout, 46 µg/mL in guppies, 100 µg/mL in carp, and 100 µg/mL in goldfish in 96-hour tests [21,22]. The calculated LOD and LOQ of the two BMM analyses are both lower than all the stated LC_{50} for the different aquatic species, implying that both BMM analyses may be applied in detecting toxic levels of methamidophos in water.

The difference in the linearity between the two BMM analyses may be attributed to each of the cameras' International Standards Organization (ISO) performance, the measure of the light sensitivity of a camera, since there is an interrelation between light sensitivity and overall image quality [18]. A higher ISO value indicates a more sensitive image sensor and this increases the probability of taking images under low light settings, which is preferable in the BMM analyses [23]. In comparing the two BMM analyses it was also observed that their respective measured light intensity differences for the same pesticide concentration were different. They were found to be statistically different from one another at both intervals using an unpaired t -test at 95% confidence level.

This means that the linear equation and the identified pesticide concentration intervals would vary for every different camera or device that would be used for the BMM analyses.

In addition, the difference in the appearance of the glow in the photos taken using the cameras may be attributed to their differences in image quality factors, such as aperture, shutter speed, and ISO number [24]. These may have also contributed to the difference in the light readings of both BMM analyses.

4 Conclusions

The study successfully shows that the light output of the TCPO-Rubrene- H_2O_2 CL system has an inverse relationship with the methamidophos concentration, indicating that the proposed method is capable of detecting methamidophos in water samples. From the highly linear trends obtained from both the fluorescence spectroscopy and the BMM analyses, the results acquired from the proposed methods could be used in further studies, such as in the detection of pesticide residues in actual water samples of unknown pesticide concentration. Selectivity studies of the proposed chemiluminescent system with other similar organophosphate pesticides should also be performed. The BMM analyses using both the digital camera and the smartphone camera had good linearity at 100-10000 µg/mL, suggesting that they could be used as alternatives to the fluorescence spectrometer at this range; however, only the latter can be considered as a better substitute in the 1-100 µg/mL range due to its better ISO performance. The BMM analyses were also considered to be of good sensitivity, with the BMM analysis using the smartphone camera having a computed LOD and LOQ of 1.0 µg/mL and 3.0 µg/mL, respectively, and the BMM analysis using digital camera having a computed LOD and LOQ of 1.6 µg/mL and 5.0 µg/mL, respectively.

Conflict of Interest: The authors stated no conflict of interest.

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