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Resonance Rayleigh Scattering, Second-Order Scattering and Frequency Doubling Scattering Methods for the Determination of Malachite Green in Aquatic Products

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In presence of the HOAc-NaOAc buffer solution and heating condition, leucomalachite green (LMG) can reacts with I_3^- to form an ion-association complex. It made a significant enhancement of resonance Rayleigh scattering (RRS), second-order scattering (SOS) and frequency doubling scattering (FDS). The increments of scattering intensity (ΔI) were directly proportional to the concentration of leucomalachite green in a certain range. The detection limits were 1.2 ng mL^{-1} for resonance Rayleigh scattering method, 1.5 ng mL^{-1} for second-order scattering method and 1.5 ng mL^{-1} for frequency doubling scattering method, respectively. This result indicates that these methods exhibited high sensitivities. And they were simple and feasible. Three new highly sensitive and simple methods of determination leucomalachite green have been developed. With the reducing action of potassium borohydride, over 85 % of malachite green (MG) can be converted to leucomalachite green. So these can be applied to determine the malachite green in aquatic products.

Key Words: Malachite green, Resonance Rayleigh scattering, Frequency doubling scattering, Determination, Aquatic Products.

INTRODUCTION

Malachite green (MG) is a cationic triphenylmethane dye, which is used as a kind of stain in the textile industry. It also has been used as anthelmintic, bactericide and preservative in commercial aquaculture since 1936[1]. Malachite green can metabolically reduce to lipophilic leucomalachite green (LMG) being absorbed by aquatic commercial animals such as fish, shrimp and crab with a long residence time (about 40 days) in certain fatty fish tissues. A similar persistent accumulation of leucomalachite green was documented in mammalian system[2]. Therefore the majority of persistent residues present in fish are in the form of leucomalachite green[3]. A large number of studies have reported that malachite green and leucomalachite green are mutagenic, teratogenic and carcinogenic[4]. Due to this reason, malachite green is not permitted for using as an aquaculture veterinary drug in the United States, Europe and Canada. Regardless of these, the abusing of malachite green in commercial fisheries has continued worldwide owing to its low cost and ready availability.

In order to safeguard people's life safety, more analytical methods are needed to monitor the low levels of these substances in aquatic products. The European Commission requires that methods be able to determine the total quantity of malachite green and leucomalachite green residues at the minimum performance limit of 2 ng·g^{-1} and 1 ng·g^{-1} under current U.S. Food and Drug Administration sample testing protocols. Currently, many detection methods of malachite green and leucomalachite green rely on their chemical properties such as a strong chromophore at 618 nm and positive charge. Then, they combine high performance liquid chromatography with visible absorption (HPLC-VIS)[5] or mass spectrometric detection (HPLC-MS)[6]. Compared with various pretreatment methods, the use of a lead oxide column reactor to convert leucomalachite green to malachite green could quantify the residues in fish tissue[7] at or below 2 ng g^{-1}. However, the method may be plagued by some problems in this process, including rapid depletion and peak broadening, which lead to a decrease of the sensitivity. Meanwhile, these general methods need expensive instruments operated by well-trained analysts and the preparation of samples is time-consuming and not ideal for detecting a large number of samples timely[8-13]. Furthermore, some new detection methods have been established in recent years. Some have built a method of enzyme-linked immunosorbent assay (ELISA) for detecting malachite green and leucomalachite green against the antibodies. Though, enzyme-linked immunosorbent assay is simple, rapid and specific, the sensitivity of this method is not excellent. The

detection limit is established at the level of 0.05 μg L^{-1} for both malachite green and leucomalachite green[14]. So, it is necessary to develop a more sensitive, simple, rapid and selective method for the determination of malachite green and leucomalachite green.

Recently, resonance Rayleigh scattering (RRS) has been reported as a new analytical technique applied in different fields such as the analysis of biomacromolecules, organic compounds, pharmaceuticals and inorganic ions[15,16]. However, it has not been reported in detection of the total quantity of malachite green and leucomalachite green in aquatic products by now. In studying resonance Rayleigh scattering of the systems, when the emission wavelength is at double or half of excitation wavelength, the strong scattering of light will be observed. As the former is well known as second-order scattering (SOS), the latter is called as frequency doubling scattering (FDS) or hyper Rayleigh scattering (HRS). Since 1995, we have already studied them as a new spectroscopic phenomenon. A large number of researches indicated that the two kinds of scattering were the non-linear optical phenomena produced by some substances (*e.g*. ion-association complex). So, they have been applied in the study of nanoparticles and inorganic ions, biological macromolecules and some physical chemistry parameters successfully and effectively[17]. In this paper, the result shows that using potassium borohydride (KBH_4) as a reducing agent, malachite green could be converted to leucomalachite green at the proportion of over 85 %. This process can be validated by the high-performance liquid chromatography (HPLC). The total content of malachite green and leucomalachite green can be determinated under the HOAc-NaOAc buffer solution and heating condition. Leucomalachite green exists as a positive ion which reacts with I_3^- to form an ion-association complex by virtue of hydrophobic force and van der Waals force. This results in a significant enhancement of resonance Rayleigh scattering and non-linear scattering such as second-order scattering and frequency doubling scattering. In this work the chromatogram of malachite green converted to leucomalachite green, the resonance Rayleigh scattering, second-order scattering and frequency doubling scattering spectra and the reaction conditions and the influencing factors are investigated. The result indicates that the three scattering methods exhibit high sensitivities. Compared with HPLC, it is operated more simple and feasible.

EXPERIMENTAL

100 μg mL^{-1} standard stock solution of leucomalachite green (Tokyo Kasei Kogyo Co., Ltd.) was prepared with acetonitrile. The working solution of 10 μg mL^{-1} was prepared by diluting the stock solution.

20 g L^{-1} KBH_4 was prepared by dissolving 2.0 g potassium borohydride with 20 mL KOH solution (5 g L^{-1}) and diluting it to 100 mL with doubly distilled water. 0.125 mol L^{-1} NH_4OAc buffer solution was prepared by dissolving 4.82 g NaOAc and adding HOAc to pH = 4.5.

The acetonitrile was chromatographically pure. 1.0×10^{-3} mol L^{-1} of I_3^- was prepared by putting 0.1270 g I_2 in the solution which was dissolved with 2.50 g KI and diluting it to 500 mL.

The HOAc-NaOAc buffer solution was prepared with acetic acid (0.2 mol L^{-1}) and sodium acetate solution (0.2 mol L^{-1}).

All the reagents used were of analytical reagent (A.R.) grade and doubly distilled water was used throughout.

General procedure: Into a 10 mL calibrated tube with plug were added 1.0 mL of HOAc-NaOAc buffer solution, certain amount of leucomalachite green solution and 0.5 mL 1.0×10^{-3} mol L^{-1} I_3^-. The mixed thoroughly solution was heated in a water bath for a period of time, being cooled to room temperature and diluted to the mark. The resonance Rayleigh scattering spectra was recorded with synchronous scanning at $\lambda_{em} = \lambda_{ex}$ and the second-order scattering and frequency doubling scattering spectra were recorded by scanning at $\lambda_{em} = 2\lambda_{ex}$ and $\lambda_{em} = 1/2\lambda_{ex}$, respectively. The scattering intensity I_{RRS}, I_{SOS} and I_{FDS} for the reaction system and I^0_{RRS} , I^0_{SOS} and I^0_{FDS} for the reagent blank at their maximum wavelengths were measured, $\Delta I = I\text{-}I^0$, the absorption spectra were recorded simultaneously.

Sample preparation: The following was the pretreatment process. A 5 g fresh fish tissue was treated with 20 mL mixture of acetonitrile and 0.125 mol L^{-1} ammonium acetate buffer solution (pH = 4.5, v/v = 1:1) and 1 g neutral Al_2O_3 and was centrifuged at 5000 rpm for 10 min. Then, 1 mL KBH_4 should be added in the collected supernatant. The reacted solution was extracted twice with 1 mL chloroform. At last all the chloroform solution was fixed to 10 mL with acetonitrile and got ready for determination.

RESULTS AND DISCUSSION

HPLC spectrum of malachite green converted to leucomalachite green: The chromatographic column was C_{18} (4.6 mm × 250 mm *i.d*, 5 μm), mobile phase was CH_3CN and HOAc + NH_4OAc buffer solution (80/20 = v/v), detection wavelength 267 nm, flow rate 1.0 mL min^{-1}, column temperature 25 ºC and the added quantity 50 μL. Under this condition, the absorption peak of leucomalachite green occurred at 5.22 min showed in Fig.1 (a) When malachite green is reduced by the KBH_4, there is a peak at 5.22 min, too [showed in Fig.1 (b)]. While the proportion of KBH_4 and leucomalachite green (1 mg L^{-1}) was 2:1 (v/v), the transformation was over 85 %. This result was calculated by the areas of the absorption peak.

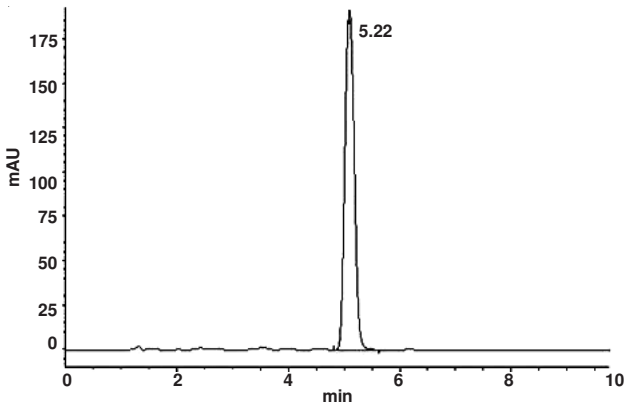


Fig. 1(a). High performance liquid chromatogram obtained from the standard solution of leucomalachite green at 4 mg L^{-1}

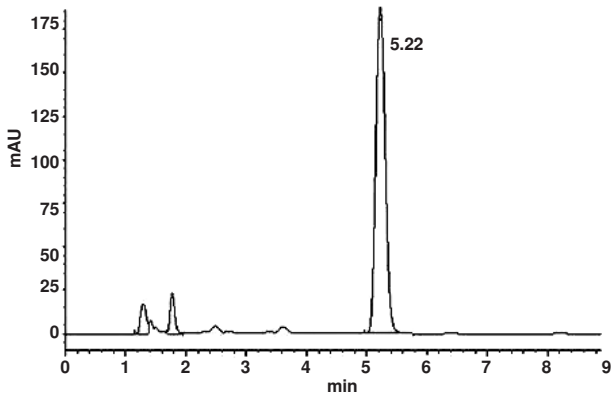


Fig. 1(b). High performance liquid chromatogram obtained from the standard solution of malachite green at 4 mg L^{-1}, which was converted to leucomalachite green by the potassium borohydride

Resonance Rayleigh scattering spectrum: The resonance Rayleigh scattering spectrum of the system was showed in Fig. 2. It could be seen that leucomalachite green has faint resonance Rayleigh scattering peaks under the optimum conditions. When I_3^- was added in the system, the resonance Rayleigh scattering intensities were enhanced greatly. The peaks were located at about 474, 484 and 494 nm, according to Fig. 2. It was also clearly showed that the resonance Rayleigh scattering intensity (ΔI_{RRS}) at wavelength of 474, 484 and 494 nm was directly proportional to the concentrations of leucomalachite green. So, the resonance Rayleigh scattering method can be applied to determinate leucomalachite green.

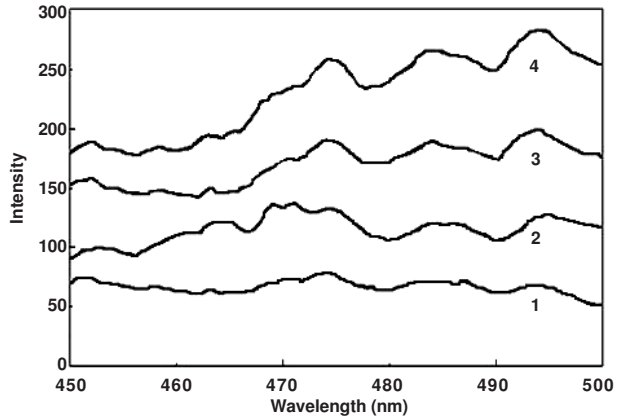


Fig. 2. Resonance Rayleigh scattering spectra of the leucomalachite green-I_3^- systems

Second-order scattering and frequency doubling scattering spectrum: Figs. 3 and 4 showed the spectrum of second-order scattering and frequency doubling scattering for the leucomalachite green-I_3^- systems. λ_{ex} was the excitation wavelength and λ_{em} was the emission wavelength. The intensities of second-order scattering and frequency doubling scattering were changed with the difference of excitation wavelengths. When $\lambda_{ex}/\lambda_{em}$ was 320/640 nm, the intensity of second-order scattering has reached to the highest and when $\lambda_{ex}/\lambda_{em}$ was 640/320 nm, the intensity of frequency doubling scattering has also reached to the highest. In this condition, the two kinds of scattering intensities (ΔI_{SOS} and ΔI_{FDS}) were directly proportional to the concentrations of leucomalachite green in a certain range.

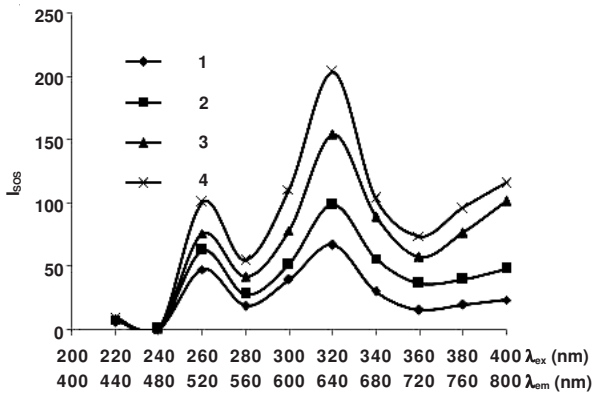


Fig. 3 Second-order scattering spectra of the leucomalachite green (LMG)-I_3^- systems

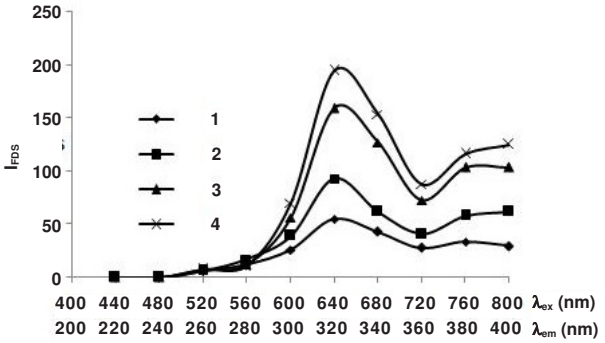


Fig. 4. Frequency doubling scattering spectra of the leucomalachite green (LMG)-I_3^- systems

Sensitivities of the methods: Through constructing the calibration graphs of ΔI *versus* the concentrations of leucomalachite green, the linear ranges, correlation coefficients and detection limits of resonance Rayleigh scattering, second-order scattering and frequency doubling scattering methods for leucomalachite green were investigated. The results showed that the linear ranges were 0.004 - 0.1 μg mL^{-1} for the resonance Rayleigh scattering method, 0.005 - 0.6 μg mL^{-1} for the second-order scattering method and 0.005 - 0.2 μg mL^{-1} for the frequency doubling scattering method. The lowest detection limits (3s) were 1.2 ng mL^{-1} for the resonance Rayleigh scattering method, 1.5 ng mL^{-1} for the second-order scattering method and 1.5 ng mL^{-1} for the frequency doubling scattering method, respectively. (Table-1). Among them, the resonance Rayleigh scattering method had the highest sensitivity.

TABLE-1
PARAMETERS OF CALIBRATION GRAPHS FOR THE DETERMINATION OF LMG

Method	Regression equation C(μg mL^{-1})	Correlation coefficient, (r)	Detect limit (ng mL^{-1})	Linear range (μg mL^{-1})
RRS	ΔI=801.84C-17.305	0.9991	1.2	0.004-0.1
FDS	ΔI=87.111C-2.8683	0.9993	1.5	0.005-0.6
SOS	ΔI=410.05C-21.552	0.9962	1.5	0.005-0.2

Then, following was the study of the optimum conditions including influencing factors, effects of coexisted substances and analytical applications. The reaction mechanism was also discussed.

Optimum conditions for the reaction

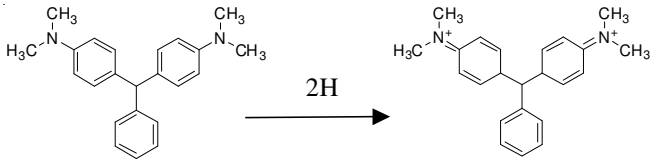
Effect of acidity: The effects of the pH value of HOAc-NaOAc buffer solution on the three methods of the leucomalachite green-I_3^- systems were investigated. The results revealed that the optimum pH value were 5.2 (resonance Rayleigh scattering system), 4.8 (frequency doubling scattering system) and 4.8 (second-order scattering system). So, the corresponding HOAc-NaOAc buffer solution was chosen as a reaction medium.

Effect of reagents concentration: The maximum intensities of scattering may be influenced by the concentration of I_3^-. Therefore, 0.05-1.0 mL I_3^- (1.0×10^{-3} mol L^{-1}) was chosen as the experimental concentration for the systems. The optimum volume of I_3^- was about 0.5 mL. Under this condition, the ΔI_{RRS}, ΔI_{FDS} and ΔI_{SOS} of the system didn't decrease too high due to the reagent blank I^0 increased.

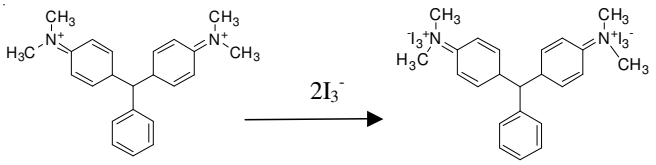
Effect of heating temperature and time of water bathing: The effects of temperature on the reaction were tested. The results showed that the optimum temperatures of reaction system were 40 ºC for 20 min (resonance Rayleigh scattering system), 25 ºC for 25 min (frequency doubling scattering system) and 25 ºC for 25 min (second-order scattering system), respectively.

Formation of the ion-association complex: Under acidic conditions, the nitrogen of leucomalachite green can be protonated[18]. The molecule of leucomalachite green existed as a cation, whereas I_3^- existed as an anion. Therefore, leucomalachite green can react with I_3^- to form an ion-association complex by virtue of the electrostatic and hydrophobic interaction. Resonance Rayleigh scattering, second-order scattering and frequency doubling scattering are both an absorption re-scattering process with equal, half and twice frequency, which can lead to the obvious enhancement of resonance Rayleigh scattering, second-order scattering and frequency doubling scattering intensity[19]. This resonance enhancement effect, the increase of this molecular volume and hydrophobicity of the ion-association complex were the main reason for the enhancement of resonance Rayleigh scattering, second-order scattering and frequency doubling scattering. Under the experimental condition, the reaction of anionic I_3^- with cationic leucomalachite green by virtue of electrostatic and hydrophobic interaction forces to form ion-association complex results in the increase of molecular weight and volume as well as the enhancement of hydrophobicity, which greatly enhance the resonance Rayleigh scattering, second-order scattering and frequency doubling scattering intensity.

Eqn.1



Eq.2



Selectivity and analytical application of the methods

Selectivity of the methods: Under the optimum conditions, the effects of some coexisting substances on the determination of leucomalachite green (0.1 μg mL^{-1}) were investigated. A large number of amino acids, saccharides, lipid, common inorganic ions (NO_3^-, Cl^- and SO_4^{2-}) and metal ions (K^+, Na^+, Mg^{2+}, Ca^{2+} and Zn^{2+}) didn't interfere with the determination.

Analytical application: The grass carp, auratus and tilapia were bought at the local market. Malachite green was not determinated in all samples. Then, the accuracy was tested by a standard addition method. The recovery was 99.06-100.74 % (Table-2).

Conclusion

Under the suitable buffer solution and heating condition, leucomalachite green can react with I_3^- to form an ion-association complex which makes a significant enhancement of resonance Rayleigh scattering, second-order scattering and frequency doubling scattering. The increments of scattering intensity (ΔI) were directly proportional to the concentration of leucomalachite green in a certain range. The detection limits were 1.2 ng mL^{-1} for resonance Rayleigh scattering method, 1.5 ng mL^{-1} for second-order scattering method and 1.5 ng mL^{-1} for frequency doubling scattering method, respectively. These methods exhibited high sensitivities, simplicity and feasibility. Three new highly sensitive and simple methods of determination leucomalachite green have been developed. Meanwhile, with the reducing action of KBH_4, over 85 % of malachite green can be converted to leucomalachite green. So these methods can be applied to determinate malachite green in aquatic products.

TABLE-2
DETERMINATION OF AQUATIC PRODUCTS

Method	Sample	Found (μg mL^{-1})	Added (μg mL^{-1})	Mean, n = 3 (μg mL^{-1})	R.S.D., n = 3 (%)	Recovery, n = 3 (%)
RRS	Tilapia	No	1.5	1.4974	0.5	99.83
FDS	Tilapia	No	1.5	1.5063	0.5	100.42
SOS	Tilapia	No	1.5	1.5111	0.5	100.74
RRS	Grass carp	No	1.5	1.4896	0.5	99.31
FDS	Grass carp	No	1.5	1.4859	0.5	99.06
SOS	Grass carp	No	1.5	1.5103	0.5	100.69
RRS	Auratus	No	1.5	1.4891	0.5	99.27
FDS	Auratus	No	1.5	1.5047	0.5	100.31
SOS	Auratus	No	1.5	1.5103	0.5	100.69

No: not detected; Mean: the average quantity for the three determinations; R.S.D: Relative standard deviation.

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