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Rapid detection of malachite green residues in fish using a surface-enhanced Raman scattering-active glass fiber paper prepared by in situ reduction method

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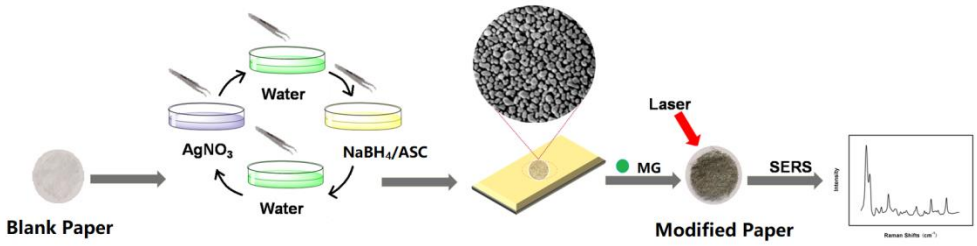
ABSTRACT

Food safety issues, especially those related to drug residues, have caused concern in recent years. Malachite green (MG), which is frequently used as ectoparasiticide, fungicide, and antiseptic in fish farming, is poisonous and carcinogenic. Several technologies and methods have been developed to accurately detect MG, but they can be time-consuming and expensive. Herein, we prepared a glass fiber paper as a surface-enhanced Raman scattering substrate for rapid and convenient detection of MG residues in fish. Uniform silver nanoparticles were deposited on the glass fiber paper by two in situ reduction steps of silver nitrate. The Raman signal of MG was detected and recorded using a portable Raman spectrometer in 10 s. There was a good linear relationship between the surface-enhanced Raman scattering signal intensity and the concentration of MG between 1×10^{-7} and 1×10^{-5} mol/L and the limit of detection was 5×10^{-10} mol/L. Residual MG in fish samples was extracted and detected with a recovery rate range of 98.9%–119.4%. This method provides a novel way to detect MG in fish.

Graphical abstract



KEYWORDS: MALACHITE GREEN, SURFAE-ENHANCED RAMAN SCATTERING (SERS), GLASS FIBER PAPER, IN SITU REDUCTION

1. INTRODUCTION

In recent decades, food safety has attracted increasing attention because of widespread concerns about chemical use [1]. Increasingly, synthetic chemicals are used as additives, coloring agents, preservatives, and decolorizers in the food industry [2]. Although some of these chemicals are highly effective in their applications, they are harmful to human health. For example, malachite green (MG) has been added to water bodies as an antibacterial agent to improve fish survival rates since 1933. However, MG has a triphenylmethane structure, which makes it highly toxic and carcinogenic[3]. In many countries, and especially China, illegal use of MG occurs frequently because it is inexpensive and is highly effective against saprolegniasis[3, 4]. The Food and Drug Administration (FDA) has listed it as a priority chemical because of its carcinogenicity[5]. Therefore, MG is highly restricted or not permitted for use as an aquaculture veterinary drug in many countries and areas, including the European Union (EU), the United States, Canada, China, and Japan. Consequently, sensitive and rapid methods that can be applied to determination of MG residues in fish are in demand.

Several methods have been developed for MG detection including high-performance liquid chromatography (HPLC)[6], gas chromatography-mass spectrometry [7], liquid chromatography-mass

spectrometry (LC-MS)[8], and the enzyme-linked immunosorbent assay (ELISA)[4]. Most of these methods are limited in their application and have some drawbacks. Although HPLC, gas chromatography-mass spectrometry, and LC-MS are sensitive and selective, they use expensive apparatus and require professional operators [9]. The existing ELISA takes around 4 h and needs a MG antibody and enzyme conjugate, which are expensive. Furthermore, the ELISA has many redundant steps, such as incubation and plate-washing. Hence, a more rapid and simpler method to detect MG residues in fish is required.

Surface-enhanced Raman scattering (SERS) is an attractive tool for highly sensitive detection of explosives[10], pollutants[11], tumor-related biomarkers[12] and pesticides[13] and uses the Raman signal of the molecule itself[14]. To date, most SERS substrates are colloids, glass slice, and anodized aluminum [3, 15-17]. Colloids are easy to prepare but unstable[18]. Glass slices and anodized aluminum are cheap and commercially scalable[19], but they are hard, fragile, and most importantly, not ecofriendly. Therefore, a strong, ecofriendly, and portable SERS substrate is required for Raman signal enhancement and real application.

Paper has recently been rediscovered as an attractive and promising substrate for inexpensive analytical tests and has attracted attention for health and environmental applications [20-23]. Chromatographic paper and filter paper have been used as SERS substrates because they are inexpensive, portable, and disposable[3, 23]. A flexible SERS substrate with nanoscale Au, Ag, or a metal composite on its surface is highly desirable for various applications because of excellent SERS signal enhancement factors (EFs)[24]. Furthermore, according to theoretical calculations, the EF for silver nanoparticles (Ag NPs) is 10^{11} and that for Au NPs is 10^{8}[25]. In most cases, the raw material used to prepare Au NPs is chloroauric acid and that used to prepare Ag NPs is silver nitrate ($AgNO_3$). In consideration of economy and efficiency, Ag NPs are better than Au NPs for use as an enhancing medium.

Here, based on previous work[26], we prepared an improved paper-based SERS substrate within 16 min using two in situ reduction steps of $AgNO_3$ on glass fiber paper. This two-steps in situ reduction method is more effective and faster than traditional methods. After soaking the modified glass fiber paper in a MG solution for 20 min, we rapidly and sensitively detected MG residues in fish samples with a portable Raman spectrometer. This method shows potential application to on-site detection of MG in aquaculture water within 1 h. Considering all these advantages, this method is promising for

application to the detection of other drug residues.

2.EXPERIMENTAL

2.1.Reagents and materials

Silver nitrate ($AgNO_3$, 99%), sodium borohydride ($NaBH_4$, 98%), MG (98%), methylene blue (MB, 98%), sodium hydroxide (NaOH, 98%), ascorbic acid (ASC, 99%) and hydrochloric acid (HCl, 36-38%) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Chloramphenicol (98%), crystal violet (90%), furazolidone (98%), and sulfadiazine (98%) were purchased from Aladdin Co. Ltd. (Shanghai, China). Glass fiber paper, polyether paper, and filter paper were purchased from Shanghai Goldbio Biotechnology Co. Ltd (Shanghai, China). Fish was purchased from a Walmart supermarket in Shanghai. Hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$, 99%), acetonitrile (99.8%), anhydrous magnesium sulfate ($MgSO_4$, 99.5%), aluminum oxide (Al_2O_3, Brockmann I) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (98%) were purchased from Sigma-Aldrich Co. Ltd. (U. S. A.). A MG Elisa Kit was purchased from Shanghai Jianglai Industrial Limited by Share Ltd. (Shanghai, China). Deionized water was purchased from Fudan University Danhua Electronics Technology Industrial Corporation (Shanghai, China).

2.2. SERS substrate preparation

First, we filled four separate wells in a six-well plate with $AgNO_3$ solution (2×10^{-2} mol/L), deionized water, $NaBH_4$ solution (2×10^{-2} mol/L), and deionized water. The plate was put on a Hy-4 Multifunctional Laboratory Shaker with an oscillation frequency of 120 rpm. Then, a circular glass fiber paper with a radius of 3 mm was soaked for 30 s in each of the four wells successively. The first reduction step was completed by repeating this process eight times.

Similarly, the second reduction step was carried out by soaking the glass fiber paper in four separate wells filled with $AgNO_3$ solution (2×10^{-2} mol/L), deionized water, ASC solution (2×10^{-2} mol/L), and deionized water in a six-well plate. The plate was put on a Hy-4 Multifunctional Laboratory Shaker with an oscillation frequency of 120 rpm. The glass fiber paper was soaked in each well for 60 s. The second reduction required four cycles of the soaking process.

Finally, the modified paper was rinsed with deionized water three times to remove the reactants and stored in deionized water. The preparation process did not use tedious procedures or expensive equipment, and the whole process could be completed in less than 30 mins.

2.3. Characterization of the glass fiber paper modified with AgNPs

The optical properties of the glass fiber paper were analyzed using a UV-visible spectrometer for solid samples (Evolution 200, Thermo Fisher Scientific, USA). The morphology of the glass fiber paper coated with AgNPs was determined using a Nova NanoSEM (NPE207, Thermo Fisher Scientific).

2.4. SERS signal detection

SERS spectra were recorded using a portable Raman spectrometer (i-Raman@plus, BWS465-785s, BWTEK, Shanghai, China) and a confocal Raman system (XploRA, Horiba Jobin Yvon, France). The excitation wavelength was 532 nm, the power was 250 mW, and the integration time was 10000 ms. Pieces of the modified paper were put into a 10^{-5} mol/L MB solution or a MG solution with a concentration between 10^{-5} and10^{-7} mol/L for 20 mins. Then, they were put on the middle of a plastic plate with a hole at the center for 5 mins to dry (Fig. S1a). Raman spectra were recorded after baseline subtraction. The pH values of different MG solutions were measured using a BANTE922 pH meter (Bante Instrument Co., China).

2.5. Fish sample preparation

First, fish bought from the market was crushed and blended completely. Then, 2.0 g of the homogenized fish was added to a centrifuge tube with 500 μL of a $NH_2OH{\cdot}HCl$ solution. The centrifuge tube was kept in the dark for 10 mins. Next, 10 mL of acetonitrile and 1.0 g of $MgSO_4$ were added to the tube and it was mixed for 1 min. Finally, 4.0 g of Al_2O_3 was added to the tube and it was put on the shaker at 250 rpm for 10 mins. The supernatant was obtained after centrifuging the mixture at 8000 rpm for 5 min. Then, the sample was dried with nitrogen gas. The residue was treated with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone solution and mixed gently for 10 mins. Then, 0.5 g of Al_2O_3 was added and the solution was mixed for 1 min. The mixture was centrifuged at 12000 rpm for 5 mins and the supernatant was filtered through a 0.45-μm organic phase filter. Finally, the modified glass fiber paper was put into the solution for 20 mins and then analyzed with the method we describe above.

3. RESULTS AND DISCUSSION

3.1. Preparation and characterization of the SERS substrate

The preparation procedure of the glass fiber paper is illustrated in Scheme 1. Briefly, the paper was modified with Ag NPs by immersion in various solutions in a six-well plate, which were used to carry out two reduction steps of $AgNO_3$ with $NaBH_4$ and ASC. After deposition of the Ag NPs,

Scanning Electronic Microscopy（SEM） was used to characterize the morphology of the glass fiber paper surface. The Ag NPs were uniformly deposited on the surface (Fig. 1a). The Ag NPs ranged in size from 20 to 50 nm and were distributed in discrete groups (or "islands"), which contributed to the excellent SERS enhancement effect. An UV absorption spectrum of the modified glass fiber paper (Fig. 1b) was obtained using a UV-visible spectrometer for solid samples. The maximum absorption peak of Ag NPs deposited on the paper was at 307 nm, and no absorption peak was observed for the paper without Ag NPs. The literature about the UV absorption of Ag NPs suggests that the absorption peak of Ag NPs should be at around 400 nm[27]. The main reason for the difference between our results and the literature is that the Ag NPs on the paper used in our experiment were completely dry. The glass fiber paper might influence the results of the solid-state UV experiment. We used MB, which has obvious Raman characteristic peaks at 448 cm^{-1}, 503 cm^{-1}, 1400 cm^{-1}, and 1620 cm^{-1}, to evaluate the SERS enhancement effects of three different type papers. Figure 1c shows the Raman spectra of the glass fiber papers before and after modification with Ag NPs. The Raman signal of MB from the glass fiber paper after SERS Ag NPs modification confirmed that the glass fiber paper substrate was prepared successfully. The blank paper showed no Raman signal.

Furthermore, we evaluated glass fiber paper, polyester fiber paper, and filter paper as SERS substrates. Because the glass fiber paper had the largest interspace between fibers (Fig.S2), it had the best SERS enhancement effect among the three types of paper tested based on a comparison of the intensity of the 448 cm^{-1} peak in the Raman spectra (Fig. S3a) after immersion in the MB solution (10^{-5}mol/L).

We also investigated the effect of different numbers of reduction cycles on the Raman signal using glass fiber paper as the SERS substrate. The glass fiber paper after eight cycles of the first reduction step and four circles of second reduction step demonstrated the best Raman signal, and the conditions were selected as the optimum experimental conditions (Fig. S3b and S3c). Repeat cycles of the two reduction steps slow down the reaction between $AgNO_3$ and $NaBH_4$ or ASC, and a slower rate of reduction contributes to formation of the "Ag island" structure on the glass fiber paper.

To demonstrate the activity of the SERS substrate, the Raman spectra of the modified paper and blank paper were investigated at MG concentrations of 10^{-8} mol/L and 1 mol/L, respectively (Fig. S4). Generally, the SERS EF is acquired from the average number of absorbed molecules (N) in the scattering volume of the SERS and non-SERS areas. It is assumed that $N = cV$, where c (mol/L) is the

concentration and V (m^3) is the scattering volume. Therefore, the SERS EF of our method was calculated using the following equation[25]:

$$\mathrm{EF} = \frac{(I_{SERS})(c_{bare})}{(I_{bare})(c_{SERS})} \quad (1)$$

where c_{SERS} is the concentration of MG on the modified SERS paper and c_{bare} is that on blank paper, respectively. The Raman intensities of modified paper and blank paper at 1174 cm^{-1} were I_{SERS} = 12591.8 and I_{bare} = 965.6, respectively. The corresponding SERS EF was 1.3×10^8, which satisfied the requirement for single molecule detection. Therefore, the modified glass fiber paper substrate demonstrated superior and stable SERS activity for further practical applications.

3.2. Stability of the SERS paper substrate

Reproducibility of a SERS substrate is of critical importance for practical application to routine analysis. The substrate preparation was repeated five times to evaluate the stability and repeatability. The Raman spectra of the five paper substrates had low relative standard deviations (~10%) for the intensity of the peak at 448 cm^{-1} (Fig. 2a), which illustrated the preparation method was repeatable.

The stability of the SERS paper substrate is important in determining the method performance. Consequently, we evaluated the stability of the modified paper over time. Modified papers were stored in deionized water for different periods and then used to detect MG in aliquots of the same solution. We successfully detected MG using the glass fiber paper stored for 10 days (Fig. 2b). The modified glass fiber paper stored for 5 days showed good performance for MG detection and the decrease in intensity of the Raman signal was less than 20% compared with fresh prepared paper（Fig.2c）. These results indicate that deionized water effectively shields the Ag NPs on the glass fiber paper from oxygen or other oxidants in the environment.

3.3. Optimization of the SERS detection conditions

According to previous studies [1, 28], the pH value of a MG solution greatly affects the SERS intensity since MG will be present in oxidized and reduced states at different pH values. To evaluate the effect of pH on the SERS detection in our method, we used HCl and NaOH solutions to adjust the pH of the MG solution. Detection of MG was successful within the neutral pH range (6.86–8.33) (Fig. 3). During these experiments, the modified paper showed different degrees of color fading when put into an acidic (pH < 6.86) or alkaline (pH > 8.03) MG solution. This phenomenon could be explained by the presence of different forms of MG at different pH values, and negative effects of acidic and

alkaline MG solutions on the Ag NPs on the glass fiber paper resulting in destruction of the Ag NP's "island structure".

Previous research[29] indicates that the uniformity of dispersion of single molecules on a paper surface is related to the soaking periods. To determine the optimum soaking period, we recorded Raman spectra for papers with different soaking periods (1, 2, 5, 10, 20, 30 minutes) using the confocal Raman System. Spectra were recorded at 10 random points on the glass fiber paper for the 10^{-5} mol/L MG solution and averaged. The spectra recorded after soaking for 20 and 30 min were almost the same, and 20 min was selected as the optimum soaking period (Fig. S5). Characteristic peaks for MG were detected even after only 1 min of soaking, which suggests that this method is very sensitive and capable of detecting low concentrations of MG.

3.4. Selectivity of the developed method

To investigate interference from other substances with our method, we tested chloramphenicol, crystal violet, furazolidone, and sulfadiazine (Fig. 4). These compounds are commonly used for treatment of various diseases in fish. A 1-mL aliquot of each solution (10^{-5} mol/L) was mixed with 1 mL of a MG solution (10^{-5} mol/L) and 1 mL of deionized water. The resulting mixtures were analyzed using a portable Raman spectrometer. The spectra (Fig. 4) were obtained using the same method described above for only MG detection. The spectra (red lines in Fig. 4a–d) showed characteristic peaks for MG at 432–437cm^{-1}, 797–802cm^{-1}, 1166–1170cm^{-1}, and 1613–1617cm^{-1}. Peaks at 1170 cm^{-1} and 1615 cm^{-1} were assigned to the in-plane vibrations of ring C-H and stretching vibrations of C=C in the three phenyl rings, respectively. Peaks at around 435 cm^{-1} and 800 cm^{-1} were assigned to out-of-plane vibrations of phenyl rings and the out-of-plane motion of aromatic hydrogens respectively. A comparison between the spectrums with and without interfering compounds shows that these drugs will not interfere with the detection of MG.

3.5. Detection of standard MG solutions

To verify the feasibility of the method, we recorded the Raman spectrum of a standard MG solution using the modified glass fiber paper as a SERS substrate. Modified papers were soaked in MG solutions with different concentrations for 20 min and then were placed on a plastic plate (Fig. S1a) for 5 mins. Spectrums (Fig. 5a) were obtained using the method described above. The spectra showed characteristic peaks for MG at 432–437 cm^{-1}, 797–802 cm^{-1} 1166–1170 cm^{-1}, and 1613–1617 cm^{-1}.

The correlation coefficients (R^2) obtained by linear regression between the intensities of the peaks

at 433, 1170, and 1615 cm^{-1} and the concentrations of MG were all less than 0.9800. The correlation coefficient (R^2) between the intensity of the peak at 797 cm^{-1} and the MG concentration was 0.9899 (Fig. 5b). These results show that within the studied concentration range (10^{-5} mol/L to 10^{-7} mol/L), there is a good linear relationship between the intensity of the characteristic Raman peaks and the MG concentration. Compared with other methods[1], our method shows similar linearity and repeatability. The limit of detection (LOD) of this method is 5×10^{-10} mol/L, which is roughly equivalent to 20 ng/mL. Although our LOD is higher than that of the ELISA method (5 ng/mL)[4], HPLC method (~1 ng/g)[6], and LC-MS/MS method (3 ng/mL)[5], it is well below the detection limits specified by the FDA and EU (2 μg/mL) [6]. Compared with other commercial methods, our method is simple, inexpensive, and rapid. Therefore, the SERS paper substrate could be effectively applied to reliable analysis of the MG concentration.

We also investigated the Raman spectra (Fig. S6) of standard MG solutions detected using a confocal Raman system to verify the sensitivity of our method. Each Raman spectrum was collected at 10 random points on the paper substrate. The LOD with the confocal Raman system was 10^{-11} mol/L, and similar to the LOD in another reference[26].

3.6. Comparison with the ELISA kit method

To demonstrate the practicality of our method, we compared it with a commercial ELISA kit. Experiments were performed following the manufacturer's instructions. The correlation coefficient (R^2) of the ELISA method was 0.9967 (Fig. S7). The results for the MG solutions (10^{-5} to 10^{-7} mol/L) were read using a microplate reader (SPR-960, SUNOSTIK, China). The linear range of the ELISA was 1–80 μg/L, and the detection range given by the manufacturer was 1–250 μg/L. By comparison, the linear range of our method was 30 μg/L to 3 mg/L. Although the LOD of the ELISA method (0.88 μg/L) was lower than that of our method, the wider linear range of our method will allow for accurate detection over a wider range.

3.7. Detection of MG extracted from real fish samples

Next, we investigated the application of the SERS substrate to detection of MG extracted from fish samples. Fish were immersed in MG solutions of different concentrations overnight to simulate environmental conditions during fish growth. To eliminate the effects of other chemicals, we extracted the MG residues from the fish samples using the methods described in Section 2.5. After this treatment, modified glass fiber paper was soaked in each solution for 20 mins. Spectral analysis of the glass fiber

papers showed characteristic peaks for MG at 432–437 cm^{-1}, 797–802 cm^{-1} 1166–1170 cm^{-1}, and 1613–1617 cm^{-1} (Fig. 5c).

The concentrations of MG in the pretreated fish samples were calculated using the linear regression equation in Fig. 5b and the intensity of the 797 cm^{-1} peak. Then, we calculated recovery rates (Table 1) using the ratio of the concentration of MG detected to that of the MG in the solution. The recovery rates for samples 1 (98.9%), 2 (102.0%), and 3 (105.6%) were quite good compared with recovery rates (80%–120%) reported in the literature[1, 3, 30, 31]. The method described here is also cheaper and simpler than the other reported methods. For samples 4 and 5, the recovery rates were quite far from 100%, and the MG concentration was close to the LOD of the method. However, semi-quantitative results could be obtained. Sample 7 was used as a control, and no MG was added to the solution that the fish sample was soaked in. The relative standard deviations of these samples are shown in Table 1. It is worth noting that methods reported by Masoumeh[9] and Ju[32] achieved recovery rates ranging from 95.6% to 104.3% and 96.4% to 106%, respectively. Compared with these earlier results, our method shows similar recovery rates in real samples and could be applied to the analysis of fish farming.

4. CONCLUSIONS

We developed a rapid and facile method to detect MG in fish using a SERS substrate prepared by two in situ reduction steps of $AgNO_3$ on the surface of a glass fiber paper. The SERS substrate could be prepared within 30 min. The modified paper prepared by this method has a shelf life of at least 7 days (Fig.2), which means that it can be prepared in advance. This glass fiber paper substrate is convenient and has good sensitivity for MG detection using a portable Raman spectrometer. There is a linear relationship between the intensities of the Raman peaks of MG and the MG concentration between 10^{-5} and 10^{-7} mol/L and the LOD is 5×10^{-10} mol/L. Although the LOD of this method is not as low as that of another recently developed method[26], the LOD does not restrict the application of this method because 5×10^{-10} mol/L is much lower than the detection limits specified by the FDA and EU. Furthermore, this method is rapid, simple, and inexpensive and can be successfully applied to detection of MG in fish and aquaculture water. In future, more extensive studies could be performed to lower the LOD and to extend the application of this method to the detection of chemical residues in other situations, such as fruits.

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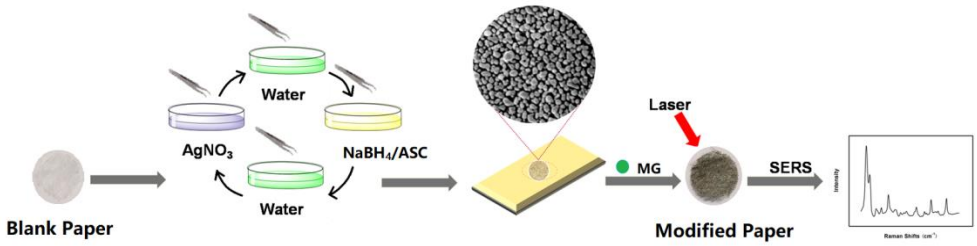

Scheme1. Scheme of substrate preparation and detection of malachite green via glass fiber paper-based SERS method.

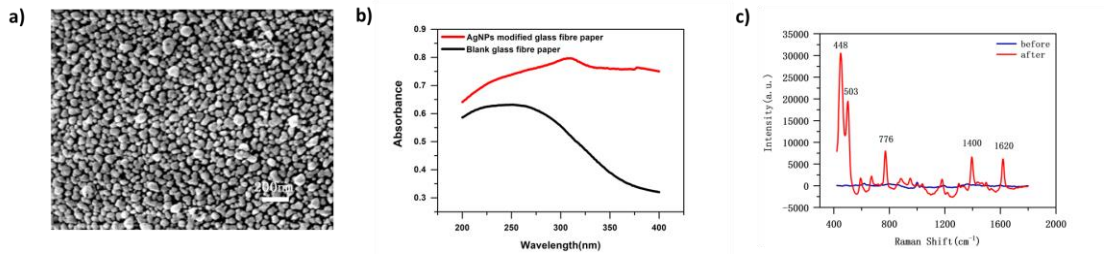

Fig.1. (a) SEM image of glass fiber paper after two-step in situ reduction of $AgNO_3$ (b) UV-visible absorption spectra of blank glass fiber paper (black) and AgNPs modified glass fiber paper (red) (c) Raman spectra of modified glass fiber paper before and after soaking in MB solution

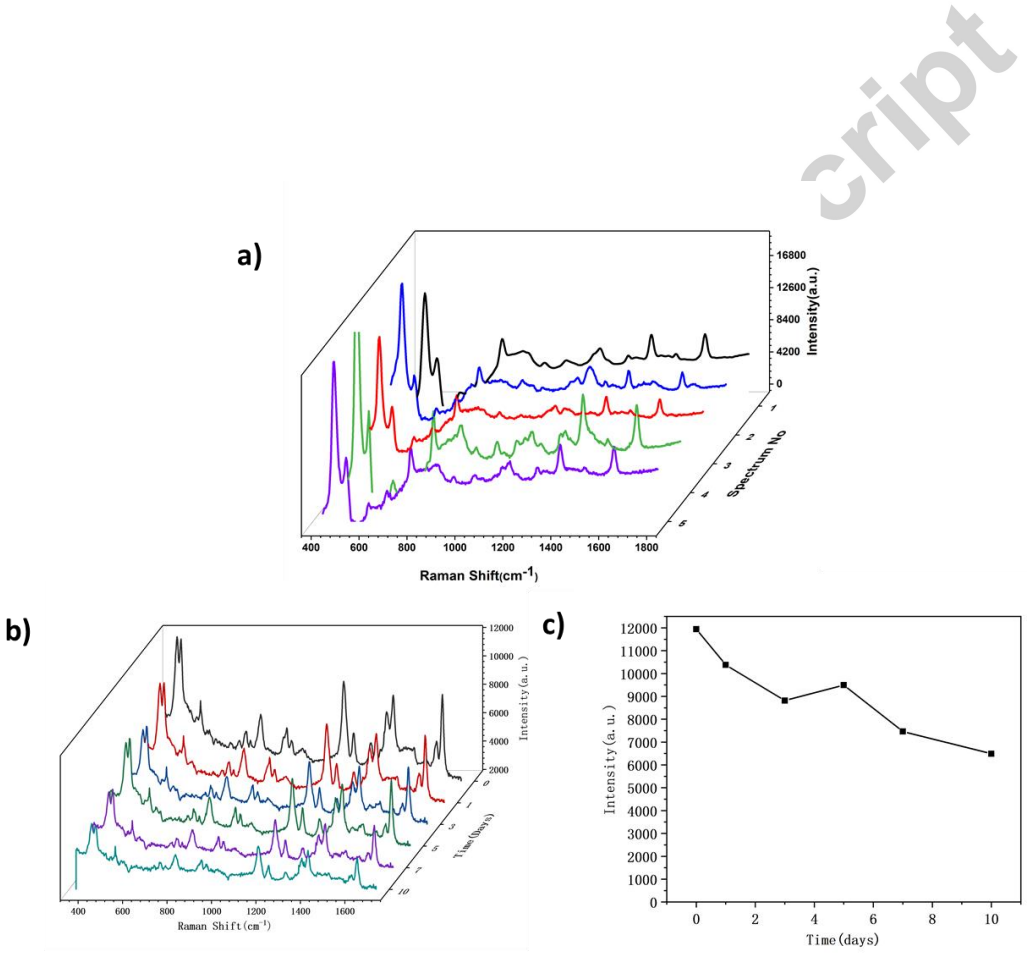

Fig.2. (a) Raman spectra of five modified glass fiber paper soaked in the same MB solution (b) Raman spectra of modified glass fiber paper which were kept in deionized water for different time (0, 1, 3, 5, 7, 10 days) and soaked in the same MG solution (c) Raman peak intensities at 441 cm^{-1} after different time of preservation in deionized water

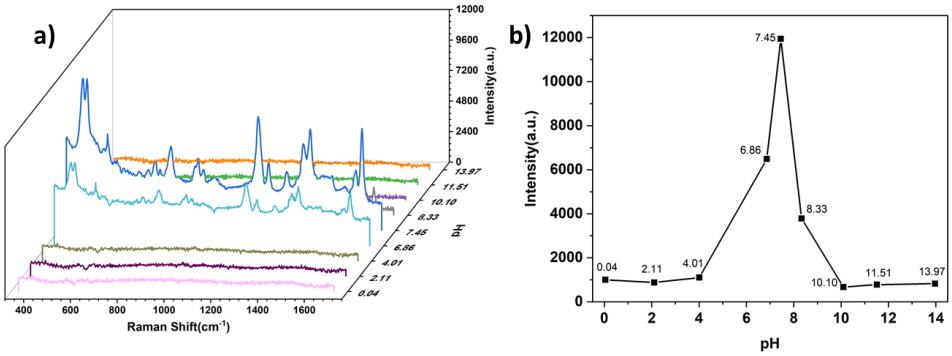

Fig.3. (a) Raman spectra of modified glass fiber paper which were soaked in the MG solution of different pH range from 0 to 14 (b) Raman peak intensities at 441 cm^{-1} under different pH values

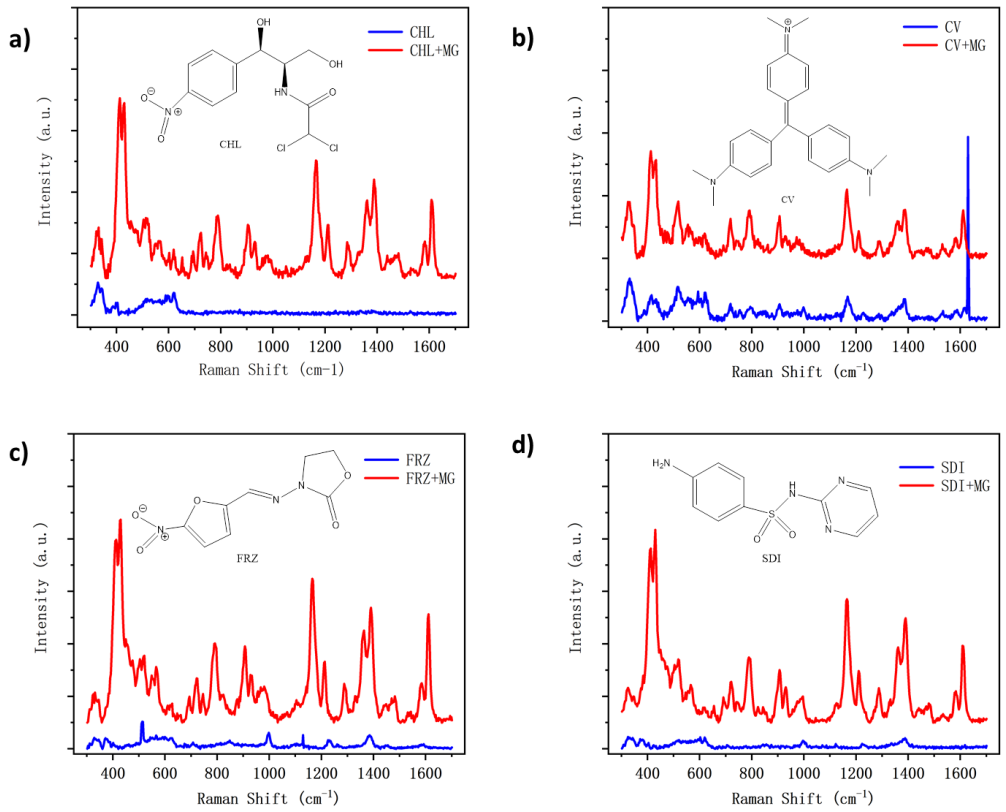

Fig.4. Raman spectrums of selectivity experiment and chemical structures of chosen pesticides (a) CHL (b) CV (c) FRZ (d) SDI (The concentration of all the detected solutions is 10^{-5} mol/L)

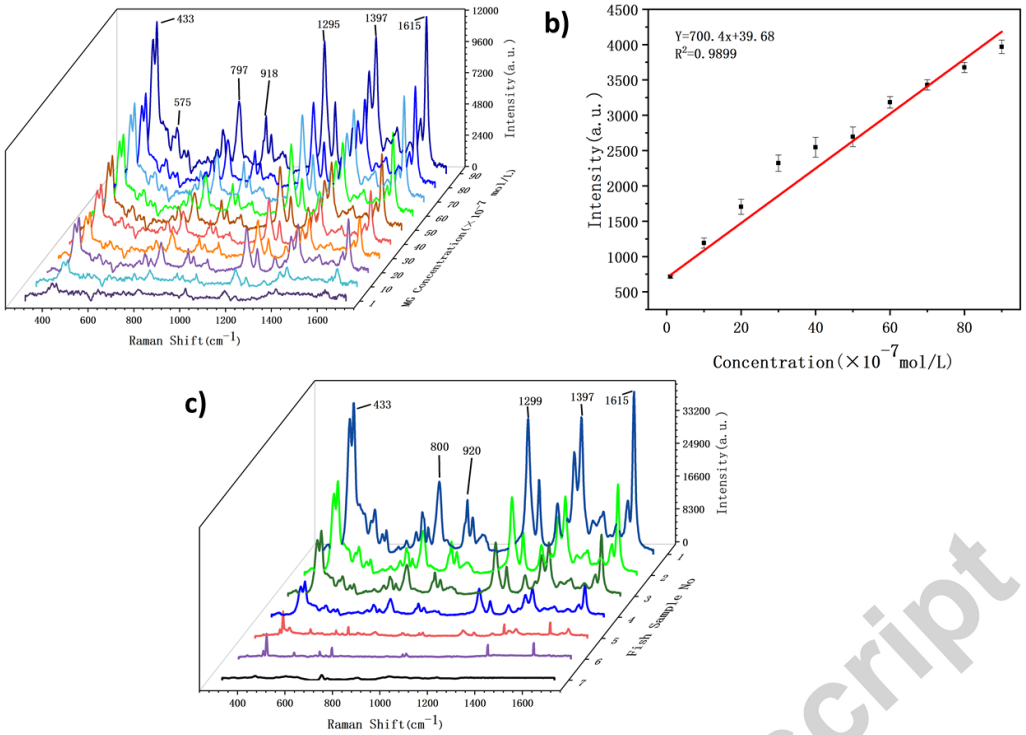

Fig.5. (a) Raman spectra of different concentrations (10^{-5}mol/L -10^{-7}mol/L) of MG in deionized water based on modified glass fiber paper (b) Correlation analysis between the intensity of peaks at 797cm^{-1} of Raman spectra and concentrations of MG solution (c) Raman spectra (1. 2.5×10^{-6} mol/L 2. 1.5×10^{-6} mol/L 3. 1×10^{-6} mol/L 4. 5×10^{-7} mol/L 5. 1×10^{-7} mol/L 6. 1×10^{-8} mol/L 7.0 mol/L) of different concentrations of MG extracted from fish sample based on modified glass fiber paper

Table1. Recovery of MG Residue in fish samples.

Group	Added(mol/L)	Found(mol/L)	Recovery (%)	R.S.D(%,n=3)
1	2.5×10^{-6}	2.472×10^{-6}	98.9	6.8
2	1.5×10^{-6}	1.530×10^{-6}	102.0	9.1
3	1×10^{-6}	1.056×10^{-6}	105.6	8.9
4	5×10^{-7}	5.867×10^{-7}	117.3	11.3
5	1×10^{-7}	1.194×10^{-7}	119.4	12.3
6	1×10^{-8}	1.471×10^{-8}	137.1	19.7
7	0	-	-	

Highlights:

1. We successfully developed an in situ reduction method to deposit uniform silver nanoparticles (Ag NPs) on glass fiber paper for the rapid and convenient SERS detection of malachite green (MG).

2. Based on this new SERS glass fiber paper substrate and the portable Raman spectrometer, a good calibration curve was obtained in the MG concentration range from 1×10^{-7}mol/L to 1×10^{-5}mol/L and the limit of detection (LOD) is 5×10^{-10} mol/L, which also showed powerful detection performance in fish sample. After comparison with Elisa method, we believe that this method can be utilized in the future.