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Determination of Malachite Green Residues in Fish Using a Highly Sensitive Electrochemiluminescence Method Combined with Molecularly Imprinted Solid Phase Extraction

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ABSTRACT: An electrochemiluminescence (ECL) inhibition method combined with molecularly imprinted solid phase extraction (MISPE) was developed for quantitative determination of malachite green (MG) residues in fish. It was found that MG could strongly inhibit the ECL signal of luminol. Under the optimized conditions, the quenched ECL intensity versus the logarithm of the concentration of MG was in good linear relationship over a concentration range from 20 to 5000 ppt. The method detection limit was found to be about 6 ppt. Molecularly imprinted polymers (MIPs) were synthesized as solid phase extraction (SPE) sorbents, and MISPE was used for the selective extraction and purification of MG. By carrying out the oxidation reaction with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), which could convert leucomalachite green (LMG) into MG, this method was successfully applied to determine MG residues in fish. A possible mechanism for the quenching effects of MG on luminol was also proposed.

KEYWORDS: electrochemiluminescence (ECL), molecularly imprinted solid phase extraction (MISPE), malachite green, leucomalachite green, luminol, fish

INTRODUCTION

Malachite green (MG) is a triphenylmethane dye (Figure 1A). It was extensively used in fish farming industries, due to its low cost, ready availability and good efficiency in resistances to important protozoan and fungal infections.[1] However, recent studies have shown that MG is a potential carcinogen, teratogen and mutagen.[2,3] Leucomalachite green (LMG, Figure 1B) could be formed by the metabolic reduction of MG, and is considered even more hazardous than MG because their degradation paths are similar but the half-life of LMG is much longer than that of MG.[4] 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), United States, Canada, China and Japan, etc. Additionally, it is demanded that sensitive methods that can be used for the determination of MG residues in fish muscle must be able to meet a minimum required performance limit of 2 ppb for the sum of MG and LMG.[5]

Chromatographic methods are currently the main ones used for the determination of MG and LMG in fish.[6−18] They are sensitive and selective, but expensive apparatus and professional operators are required. The most common sample preparation methods for the extraction of MG residues from fish muscle and cleanup are the combination of liquid/liquid partition and solid phase extraction (SPE). To obtain better specificity, selectivity and recovery, molecularly imprinted solid phase extraction (MISPE) using molecularly imprinted polymers (MIPs) as selective sorbents for a given target analyte MG in solid phase extraction (SPE) procedures was used in recent years.[15−18] It could recognize and bind the desired molecular target MG with a high affinity and selectivity, and allow MG to be eluted from cartridges almost free of coextracted compounds.

Due to its inherently high sensitivity, more and more attention was paid to the development of electrochemiluminescence (ECL) methods, in which the luminol system is the most widely used.[19−21] Based on ECL inhibition of luminol, methods for the determination of various analytes at low concentrations were established, including pentachlorophenol,[22] bisphenol A,[23] vitamin C,[24] dopamine,[25] catechol derivatives,[26] gallic acid [27] and tannic acid,[28] etc. However, the application of this method is limited by its poor selectivity. Undoubtedly, it will be very promising if it is combined with a selective sample preparation method. To our best knowledge, neither an ECL method combined with MISPE nor an ECL method for the determination of MG has been reported up to now.

In this work, the inhibition effect of MG at low concentrations on the ECL of luminol was investigated. Benefiting from the high affinity and selectivity of MISPE, a simple, low-cost and highly sensitive method for the determination of MG residues in fish was developed. The inhibition mechanism was also discussed.

MATERIALS AND METHODS

Chemicals and Reagents. Malachite green oxalate salt (MG), leucomalachite green (LMG), 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and aluminum oxide (chromatographic grade, WN-3, Neutral) were purchased from Sigma-Aldrich (Steinheim, Germany). Luminol was obtained from Sigma (St. Louis, MO, USA). Methacrylic acid (MAA) was obtained from Tianjin Kermel (Tianjin, China). Hydroxylamine hydrochloride, 2,2′-azobisisobutyronitrile (AIBN), *p*-toluenesulfonic acid, ammonium acetate and acetic acid were purchased from China National Medicines Co. Ltd. (Shanghai, China). Ethylene glycol dimethacrylate (EGDMA) was purchased from Fluka (Buchs,

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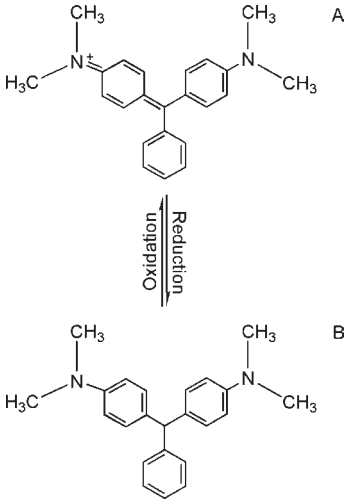

Figure 1. Chemical structures of MG (A) and LMG (B).

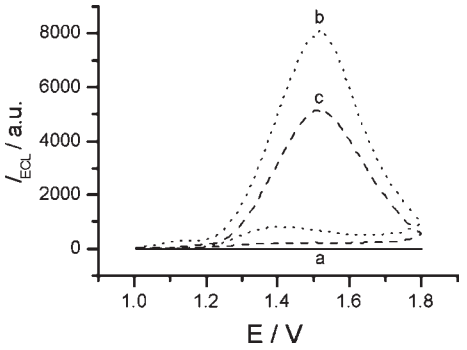

Figure 2. I_{ECL}—E curves of 5.0×10^{-10} mol/L MG (a, solid line), 1.0×10^{-6} mol/L luminol (b, dotted line) and 5.0×10^{-10} mol/L MG + 1.0×10^{-6} mol/L luminol (c, dashed line) on the GC electrode in 0.05 mol/L PBS solution (pH = 11.0). Scan rate: 0.05 V/s.

Switzerland). HPLC-grade methanol, acetonitrile and dichloromethane were supplied by Tedia (Fairfield, OH, USA). MAA and EGDMA were distilled, and AIBN was recrystallized before use. All other chemicals were of analytical grade or better, and used without further purification. Water obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used throughout. The real samples, i.e. fresh eel fillets, were purchased from local markets. The stock standard solution of luminol at a concentration of 1.0×10^{-3} mol/L was prepared in 0.01 mol/L NaOH. The stock standard solutions of MG and LMG at a concentration of 1.0×10^{-3} mol/L were prepared in acetonitrile, taking into account the content of the active substances. Working standard solutions of luminol, MG and LMG were prepared fresh daily by stepwise dilution of stock standard solutions with 0.01 mol/L NaOH and acetonitrile respectively. These solutions were stored in ambered flasks in a refrigerator at 4 °C in dark. The samples for the recovery study were prepared by adding appropriate amounts of working standard solutions into real samples just prior to use. A 0.05 mol/L phosphate buffer solution (PBS) at pH 11.0 was also prepared by mixing 0.05 mol/L Na_3PO_4 solution and 0.05 mol/L Na_2HPO_4 solution appropriately.

Apparatus. ECL measurements were carried out using a tailor-made setup consisting of a BPCL Ultra-Weak Chemiluminescence Analyzer (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China) and a CHI 660A electrochemical analyzer (Shanghai Chenhua Instrument Co., Shanghai, China), which was controlled by a personal computer. A conventional three-electrode configuration was employed, with a glass carbon electrode (∅ 3 mm) used as the working electrode, a platinum wire as the counter electrode and an Ag/AgCl (saturated KCl) electrode as the reference electrode. A tailor-made 5 mL cylindroid quartz cell was used as the ECL cell, and it was directly placed in front of the photomultiplier tube. ECL spectra could be obtained by putting a series of filters between the ECL cell and the photomultiplier tube one by one, and recording the ECL intensity successively. UV—vis absorption spectra were recorded with a Persee General (Beijing, China) TU-1901 spectrophotometer.

Preparation of MIPs. The MG-imprinted polymer was prepared following the reported methods.[15−17,29,30] Briefly, template MG (0.1 mmol) and functional monomer MAA (0.4 mmol) were dissolved in 10 mL of acetonitrile in a glass tube, prepolymerized at ambient temperature and rotated at 150 rpm for 12 h. A cross-linker EGDMA (2 mmol) and initiator AIBN (20 mg) were then added into the mixture solution. After purging with nitrogen for 10 min, the glass tube was sealed under nitrogen and then placed in a temperature-controlled water bath at 65 °C for 24 h. The resultant monolith was crushed, ground and sieved. The particles with size between 32 and 96 μm were collected, and they were washed repeatedly with methanol—acetic acid (9:1, v/v) in a Soxhlet apparatus for 72 h until MG in the supernatant could not be detected by HPLC. Finally, the particles were washed with methanol and dried at 60 °C under vacuum.

MISPE Conditions. Before extraction, the eel muscle was separated from the skin and bones, and then minced, crushed and homogenized.

The extraction method used in this study is a modification of the procedure previously reported.[17,31] An accurately weighed 5.0 g amount of the eel muscle homogenized was placed into a 50 mL centrifuge tube. 10 mL of acetonitrile, 0.5 mL of 100 g/L hydroxylamine hydrochloride, 0.5 mL of 0.1 mol/L ammonium acetate and 0.25 mL of 1 mol/L *p*-toluenesulfonic acid were added, and the sample was then homogenized for 1 min. Subsequently, 2 g of NaCl and 10 g of neutral Alumina-N were added and the mixture was vortexed for 5 min. Then, the solids were separated by centrifuging for 5 min at 4000 rpm, and the supernatant was kept. After that, all above vortexing and centrifuging steps were repeated twice, with 5 mL of acetonitrile added into the solids remaining each time. The three supernatants were combined, followed by the addition of 4 mL of 0.001 mol/L DDQ solution which could convert LMG back into MG.[17] The oxidation reaction was allowed 30 min with periodic sample agitation, and then the solution obtained was ready to load into the tailor-made SPE cartridge.

The 0.3 g amount of dry MG-imprinted polymer particles was packed into a 5 mL polypropylene column to obtain the tailor-made SPE cartridge, followed by successive preconditioning with 5 mL of methanol, 5 mL of ammonium acetate buffer solution (pH 5.5) and 5 mL of acetonitrile. 3 mL of the resulting solution mentioned above was loaded into the cartridge, and pressure was applied through it in order to remove residual solvent. Then, the cartridge was washed with 5 mL of acetonitrile, with 2 mL of acetonitrile containing 0.1% acetic acid and finally with 5 mL of dichloromethane successively. Finally, the analyte MG was eluted using 3 mL of acetonitrile containing 2% acetic acid.

The eluate was collected in a vial and evaporated to dryness under a gentle stream of nitrogen at 45 °C. The resulting residue was reconstituted for analysis in 250 μL of acetonitrile with 3 min of mixing vortex. Finally, 100 μL of the resulting solution was used for the ECL determination.

ECL Procedures. 10 μL of 1.0×10^{-4} mol/L luminol working standard solution and 990 μL of PBS were added into the ECL cell. Then, after the addition of 100 μL of the pretreated sample solution or MG standard solution to the ECL cell, the signal changes of the ECL intensity versus an applied potential (I_{ECL}—E) and the changes of current versus an applied potential (i.e., CV) were recorded simultaneously. The applied potential was controlled in the range of +1.0—1.8 V with a scan rate of 0.05 V/s. The concentration of MG was estimated

based on the quenched ECL intensity ΔI, where $\Delta I = I_0 - I_s$, and I_0 was the background ECL intensity of luminol in the absence of MG; and I_s was the intensity in the presence of a sample with MG. To eliminate the interference from dissolved oxygen, the sample solutions were degassed with argon for 15 min before taking measurements. All experiments were performed at room temperature (25 ± 1 °C) under argon. Considering the stability of MG at pH 11, each ECL determination run should be completed in 5 min.

The working electrode was polished consecutively with 1.0 μm, 0.3 μm and 0.05 μm α-Al_2O_3 aqueous slurries on a chamois leather to obtain a mirror surface before use. Prior to each run, the electrode was sonicated and thoroughly rinsed with water, and was air-dried.

RESULTS AND DISCUSSION

ECL Inhibition Behavior of MG. The control experiments showed that MG gave no ECL signals at a glass carbon electrode (Figure 2a). However, the ECL intensity of luminol under the conditions without MG (Figure 2b) was found much higher than that with MG (Figure 2c). This result may indicate that MG is able to inhibit the ECL of luminol effectively.

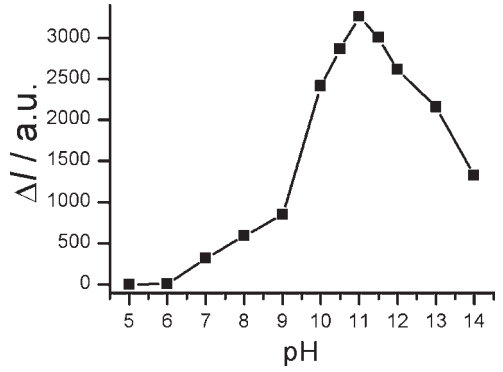

Figure 3. Effect of pH on ΔI. Experimental conditions were same as for Figure 2c.

Optimization of Experimental Conditions. The quenched ECL intensity (ΔI) was correlated with a number of factors, including the mode and scan rate of the applied potential, the buffer solution and its pH. In order to obtain a higher sensitivity of this ECL system, all these factors were optimized.

Different modes of the applied potential were tried. Results showed that good and steady ECL signal, i.e. the highest signal-noise-ratio in the ECL inhibition, was obtained using the cyclic voltammetry mode for the measurements. Different scan rates were used to examine the ECL behavior of the luminol/MG

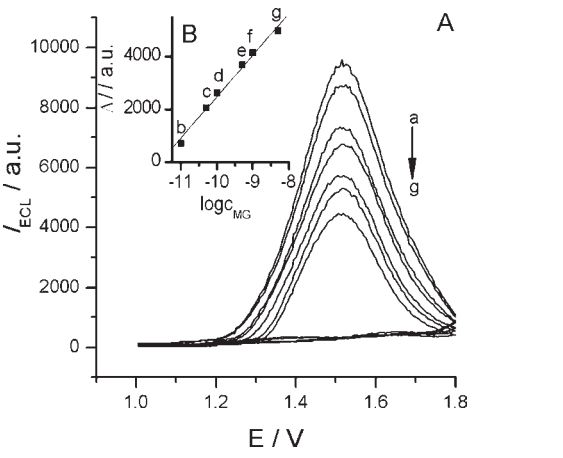

Figure 4. (A) I_{ECL}–E curves of luminol in the present of MG at the concentration of 0 (a), 1.0×10^{-11} mol/L (b), 5.0×10^{-11} mol/L (c), 1.0×10^{-10} mol/L (d), 5.0×10^{-10} mol/L (e), 1.0×10^{-9} mol/L (f) and 5.0×10^{-9} mol/L (g). (B) Calibration curve between ΔI and the logarithm of the concentration of MG. Other experimental conditions were the same as those for Figure 2c.

Table 1. Determination of MG Residues in Real Samples (Mean ± SD, n = 5)

	concn of MG residues (ppt)					
sample code	found before adding	RSD (%)	MG/LMG added[a]	found after adding	RSD (%)	recovery (%)
1	nd[b]		2000 (MG/LMG = 1/9)	1896 ± 134	7.1	94.8
			2000 (MG/LMG = 5/5)	1932 ± 108	5.6	96.6
			2000 (MG/LMG = 9/1)	1928 ± 147	7.6	96.4
2	68.5 ± 7.2	10.5	50 (MG/LMG = 1/9)	112.2 ± 10.6	9.4	87.4
			50 (MG/LMG = 5/5)	112.7 ± 8.8	7.8	88.4
			50 (MG/LMG = 9/1)	114.8 ± 7.8	6.8	92.6
3	535 ± 29	5.4	500 (MG/LMG = 1/9)	1011 ± 64	6.3	95.2
			500 (MG/LMG = 5/5)	1003 ± 76	7.6	93.6
			500 (MG/LMG = 9/1)	1008 ± 80	7.9	94.6
4	nd		20 (MG/LMG = 1/9)	17.3 ± 2.5	14.5	86.5
			20 (MG/LMG = 5/5)	16.9 ± 2.4	14.2	84.5
			20 (MG/LMG = 9/1)	17.0 ± 2.1	12.4	85.0
5	131 ± 9.5	7.2	100 (MG/LMG = 1/9)	217.5 ± 15.4	7.1	86.5
			100 (MG/LMG = 5/5)	220.7 ± 12.6	5.7	89.7
			100 (MG/LMG = 9/1)	222.3 ± 12.4	5.6	91.3

[a] To validate this method for the determination of MG residues (i.e., the sum amount of MG and LMG), three types of mixture solutions were used. MG/LMG = 1/9 means the solution was obtained by mixing MG and LMG in a mass ratio of 1:9. Similarly MG/LMG = 5/5 and MG/LMG = 9/1 indicate mass ratios of 5:5 and 9:1. [b] Not detected.

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system. It was found that the maximum ΔI value could be obtained at a scan rate of 0.05 V/s.

The ECL behavior of the luminol/MG system was investigated in different buffer media (pH = 11.0), including Tris-HCl buffer solution, $NaHCO_3-Na_2CO_3$ buffer solution and PBS buffer solution. Results showed that the ECL signal of the luminol/MG system was not found in Tris-HCl buffer solution, and unstable in $NaHCO_3-Na_2CO_3$ buffer solution. However, it was stable and strong in PBS buffer solution. In addition, the effect of pH on ΔI was also studied. No ECL signal could be found when pH < 7.0. When pH was in the range of 7.0−9.0, the ECL signal was stable but a poor ΔI could be obtained. However, ΔI increased with the increase of the pH in the range of 9.0−11.0, and then decreased with the increase of the pH in the range of 11.0−13.0, as shown in Figure 3.

Therefore, the optimal experimental conditions were selected as follows: CV at a scan rate of 0.05 V/s, and PBS buffer solution at pH = 11.0.

Calibration Curve, Linear Response Range and Detection Limit. Under the optimum conditions, the linear response range and the detection limit (LOD) for MG were measured. As shown in Figure 4, ΔI is found in good linear relationship with the logarithm of the concentration of MG over a range from 1.0×10^{-11} mol/L (3.65 ng/kg) to 5.0×10^{-9} mol/L (1825 ng/kg). The regression equation was $\Delta I = 1579.22 \log c_{MG}$ (ng/kg) + 21.87, with a correlation coefficient r of 0.9956. The instrumental detection limit was found to be 3.0×10^{-12} mol/L (1.1 ng/kg) at a signal-to-noise ratio of 3, indicating that this method is very sensitive for the determination of MG. Considering the concentration of MG would decrease about 4 times in the MISPE and ECL determination procedure, the method detection limit of MG in fish samples could be estimated to be 1.2×10^{-11} mol/L according to the established parameters, which was roughly equivalent to 5 ppt (i.e., ng/kg). It can be found that this method exhibits great advantages in the detection limit, which is approximately equal to that of the most sensitive LC−MS/MS method reported (method detection limit: 3 ppt) in the literature.[17]

Interference. In order to assess the proposed method for the analysis of MG in real samples, the interference effects of coexistence substances, which were expected to be present in the samples, were also examined. The solutions used for this purpose contain 5×10^{-10} mol/L MG and interfering species. The upper limit of an interfering species was estimated under the conditions that the relative error for determination of a standard MG solution was less than 5%. The tolerable concentration ratios were determined as follows: >100 000 for Cl^-, NO_3^-, Ac^-, CO_3^{2-}, SO_4^{2-}, PO_4^{3-}, NH_4^+, Fe^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Ba^{2+}, Ca^{2+}, Ni^{2+}, Fe^{3+}, Cr^{3+}, leucine, glutamine, methionine, cystine, hydroxyproline, tyrosine, proline, cysteine, valine, alanine, glucose, maltose, gelose; >10 000 for amitraz, chlordimeform, metronidazole, streptomycin, quinolones, penicillins, cephalosporins, sulfonamides, nitrofurans, macrolides, organophosphorus pesticides and nitroimidazole; >5000 for crystal violet, leuco crystal violet and ethyl violet. Given that fish matrix is very complex, fats and other interfering compounds that can be coextracted and give signal suppression or signal enhancement should be also considered. Fortunately, benefiting from the cleanup step developed, the MIP extraction materials with high selectivity and special ECL experimental conditions, matrix-induced effects were insignificant according to the good recoveries shown in Table 1. In a word, the anti-interference ability of this method was satisfactory, due to the efficient pretreatment of the sample.

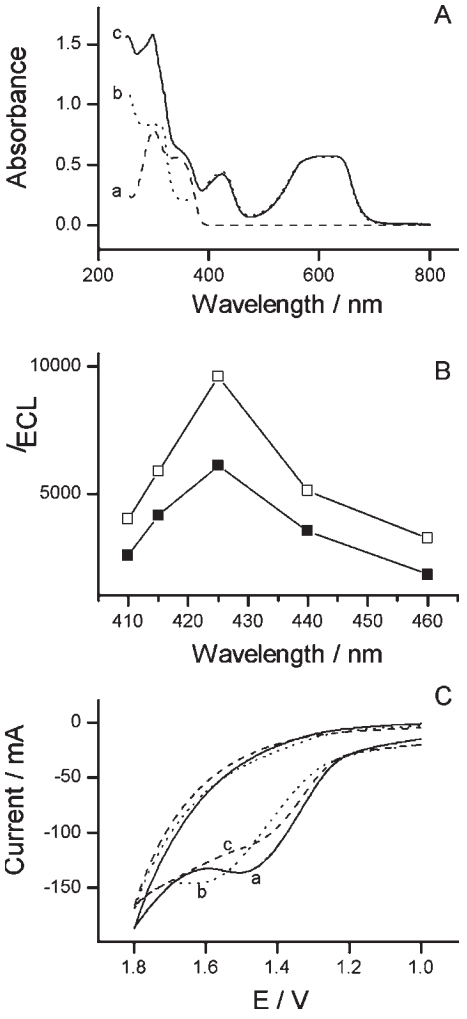

Figure 5. (A) UV−visible absorption spectra of luminol (a, dashed line), MG (b, dotted line) and luminol + MG (c, solid line). (B) ECL spectra of luminol (□) and luminol + MG (■). (C) Cyclic voltammograms of PBS (a, solid line), luminol in PBS (b, dotted line) and luminol + MG in PBS (c, dashed line).

Analytical Applications. The proposed method was validated using a series of fortified fish samples at concentrations of 20, 50, 100, 200, 500, 1000, 2000, 5000 ppt. It was found that ΔI is in good linear relationship with the logarithm of the concentration of MG in fortified fish samples over a range from 20 to 5000 ppt. The regression equation of the calibration curve was $\Delta I = 1491.58 \log c_{MG}$ (ppt) + 20.67, with a correlation coefficient r of 0.9915. LOD for the assay of MG in fish, based on 3/1 of the signal-to-noise ratio, was about 6 ppt. The quantification limit (LOQ), based on 10/1 of the signal to-noise ratio, was 20 ppt. The method was applied to quantify MG residues in real fish samples too. As shown in Table 1, good relative standard deviations (RSDs) of 5.6−14.5% and recoveries of 84.5−96.6% were obtained. Therefore, this proposed method shows potential application for the determination of MG residues in fish.

Stability of the Tailor-Made MISPE Cartridges. The stability of the tailor-made MISPE cartridges is likely to be a key factor in practical application. To test it, the cartridges were subjected to several preconditioning−loading−eluting batch operations. The

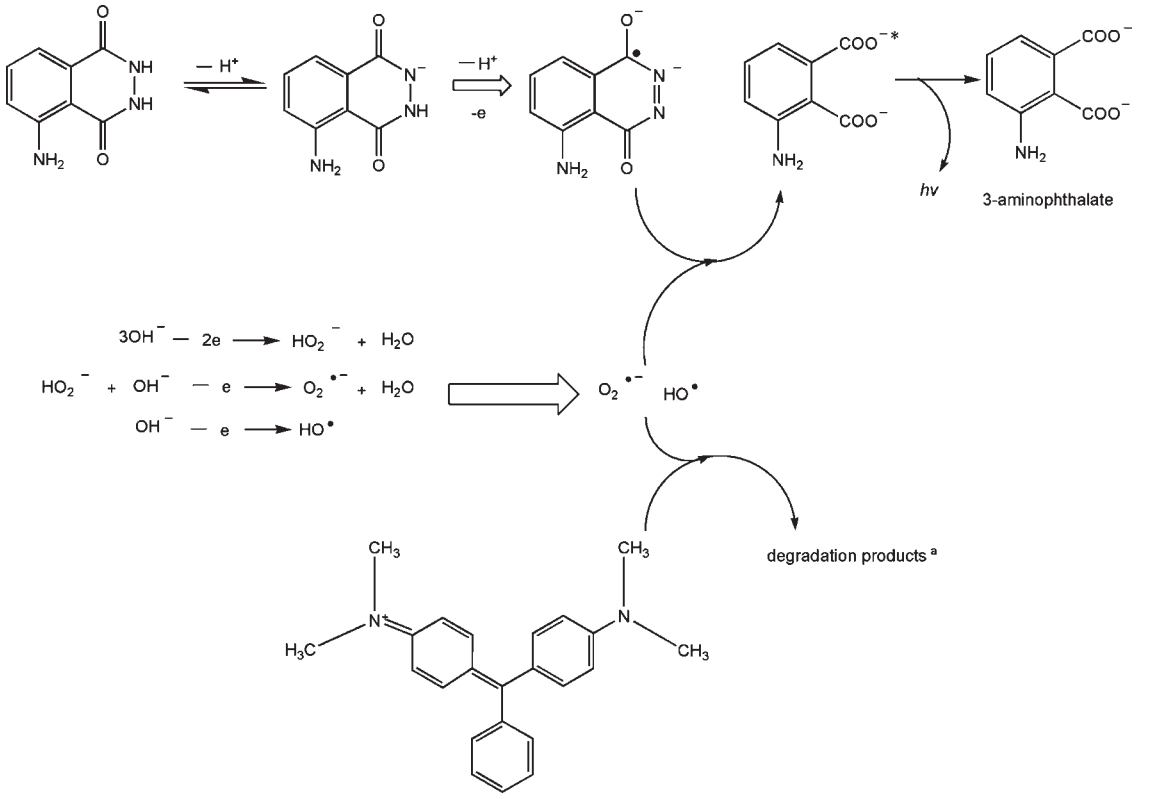

Figure 6. Mechanism for the ECL inhibition effect of MG on luminol.[36]

results showed that the tailor-made MISPE cartridge could be used at least 10 times without loss of its extraction capability, i.e., the reusability and stability of the cartridges were satisfactory.

Possible Mechanism of Inhibition. Figure 5A shows the UV−visible spectra of the system without applying electrolysis. The spectrum of the mixed solution luminol/MG was exactly the sum of that of luminol and that of MG, suggesting that no new intermediate was produced as simply mixing luminol and MG in a solution, i.e., no chemical reaction between luminol and MG can be expected.

According to the ECL emission spectra as shown in Figure 5B, the line profiles of luminol and luminol/MG were identical, and the maximum emission wavelengths were both at 425 nm, which corresponds to the light emission of excited 3-aminophthalate.[32] It indicated that ECL signals of luminol/MG system were also initiated by the ECL reaction of luminol.

A series of cyclic voltammograms is shown in Figure 5C. When MG was added into luminol solution, the oxidation peak potential of luminol shifted negatively and the peak current decreased, suggesting that the electrochemical oxidation of MG could affect the ECL inhibition of luminol greatly.

Based on our experimental results, a mechanism for the ECL inhibition of luminol/MG system was proposed as shown in Figure 6. In strong alkaline solution at a higher positive potential, OH^- was readily oxidized to $OH^•$ and HO_2^- which could be further oxidized to free radicals $O_2^{•-}$.[33,34] Free radicals $O_2^{•-}$ and $OH^•$ could react with luminol anion to generate light emission, and thus then strong ECL signals of luminol could be obtained at +1.43 V on the gold electrode,[34] at +1.54 V on the paraffin-impregnated graphite electrode [35] and at +1.50 V on the glass carbon electrode in this work. However, in the presence of free radicals $O_2^{•-}$and $OH^{•-}$, MG could be electro-oxidized, degraded and eventually converted to benzaldehyde and benzene.[36] The competition of free radicals $O_2^{•-}$ and $OH^{•-}$ between luminol and MG might result in the ECL inhibition of luminol finally.

In conclusion, an inhibited ECL method based on the luminol system combined with MISPE was successfully developed for the determination of MG. The method is simple, sensitive and accurate, and was demonstrated for the determination of MG residues in fish with satisfactory results. A possible mechanism was proposed for the explanation of the ECL inhibition behavior of this system. It was believed that the competition of free radicals $O_2^{•-}$ and $OH^{•-}$ between luminol and MG is the key factor.

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