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Detection of Subnanomolar Melamine Based on Electrochemical Accumulation Coupled with Enzyme Colorimetric Assay

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ABSTRACT: Based on the synergetic effect of the electrochemical accumulation process and the signal amplification of enzymes, a new sensitive method has been developed for the detection of subnanomolar melamine. There are two steps involved in the sensor construction process: (1) accumulation of melamine on an electrode by cyclic voltammetric method and (2) chemical coupling of horseradish peroxidase (HRP) with the accumulated melamine through the linkage of glutaraldehyde. The coupled HRP catalyzes the oxidation of guaiacol to generate an amber-colored product. Quantitative analysis of melamine is performed by measuring the absorption intensities of the colored product. Under the optimal conditions, the method showed a wide linearity in the concentration range from 1.0×10^{-11} to 1.0×10^{-8} M for melamine detection. Moreover, it has been successfully applied to detect melamine in different infant formula powders and fish feed samples.

KEYWORDS: *melamine, subnanomolar, electrochemical, colorimetric*

INTRODUCTION

Melamine (2,4,6-triamino-1,3,5-triazine) is a nitrogen-containing compound used in the production of plastics, glues, and thermoset polymers.[1] Since 1958, it had been used as a non-protein nitrogen source for cattle feed supplementation due to its high nitrogen level (66% nitrogen by mass). In 1978, however, a study concluded that melamine might not be hydrolyzed completely in ruminants.[2] From then on, melamine was not approved to be adulterated to foods or feeds, nor was it permitted to be used as a fertilizer anywhere in the world. In 2007, a series of incidents of melamine-contaminated food and animal feed occurred. Melamine contamination has been reported in a variety of food products, such as milk, infant formula, frozen yogurt, pet food, biscuits, candy, and animal feeds. Normally, proteins are the only source of nitrogen in food, so by detecting nitrogen in food, one can figure out relative protein concentrations. The Kjeldahl method[3] is commonly used to detect protein content, but it cannot distinguish between protein and non-protein nitrogen. Therefore, some unethical manufacturers illegally added melamine to food products to artificially elevate the protein content values. The ingestion of melamine could cause renal disease, bladder or kidney stones, and even death of pets and babies.[4] In order to guarantee a safe food supply and protect human health, many countries and regions such as the United States, the European Union (EU), and Australia have established a tolerance level for melamine.

The melamine issues also led to extensive and intensive scales of laboratory monitoring work. Up to now, a considerable number of techniques have been developed for the detection of melamine. Sun et al.[5] have made an extensive review of methodologies applied before 2010. The linearity and the detection limits of methods developed after 2010 for the detection of melamine are summarized in Table 1. These techniques include mass spectrometry,[6] photoluminescence,[12] high-performance liquid chromatography with UV−vis detection,[10] liquid chromatography in combination with mass spectrometry (LC-MS),[11] micellar electrokinetic chromatography,[7] surface-enhanced Raman scattering,[23] and mid- and near-infrared (MIR/NIR) spectroscopy.[24] In spite of the significant advances of those methods, melamine detection still faces some challenges. Most of the reported techniques are time-consuming, labor-intensive, and require relative expensive and complicated instrumentation.[6,7] In some scenarios a high-sensitivity method is needed because the U.S. Food and Drug Administration (FDA) stresses that baby foods should not contain any dose of melamine. Therefore, it is necessary to develop a simple, cheap, selective, and sensitive method for melamine detection. Compared to the existing methods, electrochemical sensors are advantageous due to their simplicity, low cost, and high sensitivity. Electrochemical accumulation has been employed to provide concentrations required for analysis by flameless atomic absorption spectrometry,[26] anodic stripping voltammetry,[27] and inductively coupled plasmas mass spectroscopy.[28] The electro-accumulation process enhances the analytical signal and eliminates interference from sample matrices.[29] Some attention has been paid to the electrochemical accumulation of amine-containing compounds on different electrodes.[30] Melamine is an organic compound with triamine groups, which can be accumulated on carbon electrodes by carbon−nitrogen linkage.[30] The accumulated melamine cannot be detected directly by electrochemical methods because of its poor electroactivity.[31] However, it could provide primary amine groups for the covalent attachment of proteins.[32] Horseradish peroxidase (HRP) has been extensively used as a label in enzyme-linked immunosorbent assays (ELISAs) and immunohistochemical techni-

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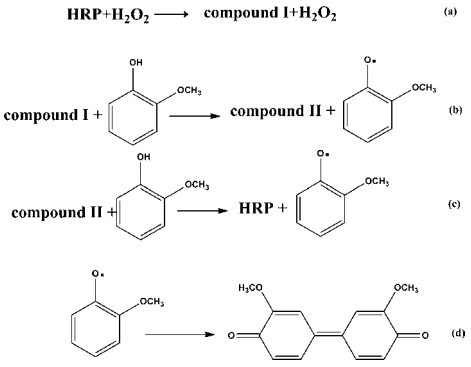
Table 1. Summary of Linearity and Detection Limits of Some Published Methods for Detecting Melamine[a]

method	linearity	DL	ref
CSI-MEKC		2.2×10^{-8} M	8
RP-HPLC	$(7.9–0.6) \times 10^{-6}$ M	79.0×10^{-8} M	9
LC	$(0.16–800.0) \times 10^{-6}$ M	4.0×10^{-8} M	10
HILC-UV	$(0–4.0) \times 10^{-6}$ M	4.0×10^{-8} M	11
HPLC-MS	$(0.004–0.8) \times 10^{-6}$ M		12
colorimetric	$(1.5–79.3) \times 10^{-6}$ M	47.6×10^{-8} M	13
fluorescence	$(0.002–50.0) \times 10^{-6}$ M	0.1×10^{-8} M	14
fluorescence	$(0.0008–0.08) \times 10^{-6}$ M	0.061×10^{-8} M	15
cELISA	$(2.0–400.0) \times 10^{-6}$ M	0.4×10^{-8} M	16
cELISA	$(0.04–1.1) \times 10^{-6}$ M		17
microfluidic-UV	$(7.9–790.0) \times 10^{-6}$ M	180.0×10^{-8} M	18
electrochemical	$(0.04–3.3) \times 10^{-6}$ M	9.6×10^{-8} M	19
electrochemical	$(1.0–66.4) \times 10^{-6}$ M	30.0×10^{-8} M	20
DPV	0.05–1.31 ppm		21
impedimetric	0.5–0.93 ppm		21
SWV	$(5–200) \times 10^{-6}$ M	800.0×10^{-8} M (98.3 ppb)	22
SERS	0.5–100 ppb	0.1 ppb	23
SERS	$(0.001–0.1) \times 10^{-6}$ M	120 ppt	24
MIR/NIR		0.76 ± 0.11 ppm	25

[a]CSI-MEKC, cation-selective injection micellar electrokinetic chromatographic method; RP-HPLC, reverse-phase high-performance liquid chromatography; LC, liquid chromatography; HILC-UV, hydrophilic interaction liquid chromatography–ultraviolet method; HPLC-MS: high-performance liquid chromatography–mass spectrometry; cELISA, indirect competitive enzyme-linked immunosorbent assay; DPV, differential pulse voltammetry; SWV, square-wave voltammetry; SERS, surface-enhanced Raman scattering; MIR/NIR, mid-/near-infrared spectroscopy; DL, detection limit.

ques[33] by using guaiacol as the substrate.[34] It catalytically achieves one electron from guaiacol at the expense of hydrogen peroxide and forms a red-brown product,[35] which is illustrated in Scheme 1:[36]

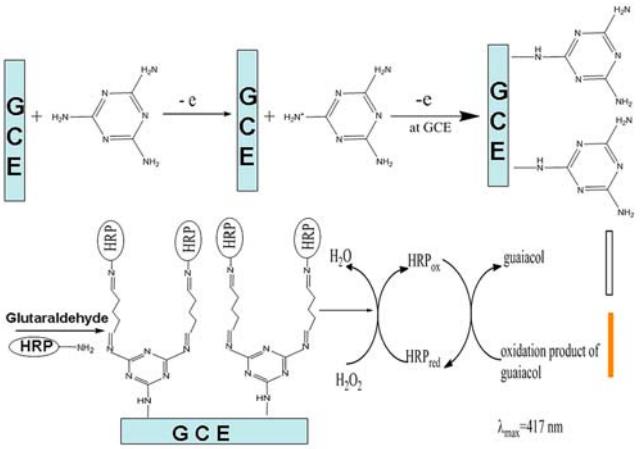
Scheme 1



In the first step, H_2O_2 is reduced by HRP with the formation of an oxidized enzyme intermediate, compound I (reaction a in Scheme 1). Compound I is rereduced in two steps. In each step, a donor substrate (guaiacol) is oxidized to give a radical product (reactions b and c in Scheme1). 3,3′-Dimethoxy-4,4′-biphenoquinone, which shows an absorption maximum around 420 nm (reaction d in Scheme 1),[37] is formed from the obtained radicals. The sensors based on enzymatic reactions of labeled HRP provide high, steady, and reproducible signal amplification. When the electrochemical accumulation process is combined with the enzymatic colorimetric amplification assay, both the advantages of electrochemical accumulation and the high catalytic performance of enzymes would contribute to increased sensitivity for the detection of melamine. Furthermore, the colorimetric assay is advantageous for screening purposes compared to the previously mentioned complex instrumental methods because of its high throughput and rapid turnaround time.[38]

In this work, a novel method for the sensitive and selective detection of melamine was developed, based on the above-mentioned concepts. This method combined the electrochemical accumulation process with enzyme colorimetric assay (EA-ECA). The sensor construction process is illustrated in Scheme 2. First, melamine is accumulated on a glassy carbon

Scheme 2. Schematic Illustration of Experimental Process



electrode by an electrochemical method. Then, by the free amine group of melamine, HRP is assembled on the electrode. The coupled HRP would catalyze the oxidation of guaiacol in the presence of H_2O_2 to generate an amber-colored product. Quantitative analysis of melamine is performed by measuring the absorption intensity of the colored product. Atomic force microscopy (AFM) was used to characterize the sensor construction processes. Factors such as electrochemical accumulation and colorimetric assay conditions were optimized. The objective of this study is to evaluate the ability of the EA-ECA technique to quantify and detect melamine in various infant formula powders and fish feed samples.

EXPERIMENTAL PROCEDURES

Reagents and Materials. Melamine, guaiacol, and H_2O_2 were purchased from Chinese Chemical Reagent Co. Ltd. (Shanghai, China). Glutaraldehyde (25%) was obtained from Merck KGaA Co. (Darmstadt, Germany). Horseradish peroxidase (HRP, EC 1.11.1.7, 300 units/mg) was purchased from Dongfeng Biological Engineering Co. (Shanghai, China) and was used without further purification. Buffer solutions with the desired pH were prepared from H_3PO_4–Na_2HPO_4. Infant formula powders (Dumex, Netherlands; Morinaga, Japan; Wyeth, Singapore) were bought from local supermarkets. A fish feed sample was bought from a local manufacturer. The melamine-contaminated fish feed sample was kindly supplied by the Jiangsu Entry–Exit Inspection and Quarantine Bureau (Jiangsu, China).

Sample Preparation. *Standard Sample Preparation.* The stock solution of melamine was prepared in water by dissolving 0.157 g of melamine in a 250 mL volumetric flask to provide a concentration of

5.0 mM. Series dilutions of the 5.0 mM standard were then prepared with 0.1 M phosphate buffer solutions (PBS), pH 3.0, to produce calibration standards from 10^{-11} to 10^{-8} M. The detection limits (3 signal/noise, S/N) were calculated according to Long and Winefordner,[39] where N was expressed as the standard deviation of noise determined in the signal domain.

Real Sample Preparation. For real sample preparation, 2.00 g of infant formula powder or fish feed sample was weighed and placed into a 50 mL poly(tetrafluoroethylene) (PTFE) centrifuge tube. Then 15 mL of 1% trichloroacetic acid and 10 mL of acetonitrile were added to precipitate proteins. After 15 min of sonication, the mixture was heated at 100 °C by a water bath for about 10 min to promote the coagulation of proteins. The mixture was centrifuged at 4000 rpm for 5 min. Then the supernatant was filtered through a 0.22 μm PTFE membrane. The effluent was collected in a 250 mL round-bottom flask, evaporated under vacuum to a small volume at a bath temperature of 40 °C, and the last solvent traces were then removed by rotating the collecting flask. The residue was dissolved with 10 mL of pH 3.0 solution for analysis. The amounts of melamine in commercial infant milk powders or fish feed samples were measured by a standard calibration method under the optimized conditions. To evaluate the recovery of the method developed, selected analytical standards were used to fortify the melamine-free extractions and detected by the EA-ECA method. To eliminate interference from the matrix, calibration curves were obtained by adding standards of melamine into extractions.

Electrochemical Accumulation of Melamine. All the electrochemical experiments were performed on a CHI 660A electrochemical workstation (Shanghai CH Instruments, China). A conventional three-electrode system was used throughout the experiments. The three-electrode system contained a glassy carbon electrode (GCE) as the working electrode (d = 3 mm, Shanghai Chenhua, China), a saturated calomel electrode as the reference electrode, and a platinum disk electrode (d = 1 mm in diameter, Tianjing Lanlike, China) as the counter electrode. Before each accumulation process, the GCEs were fine-polished with 0.3 and 0.05 μm alumna slurry on microcloth pads and then rinsed with doubly distilled water. Subsequently, they were washed ultrasonically in HNO_3 (1:1 v/v), ethanol (1:1 v/v), and doubly distilled water and allowed to dry by N_2 purge. Then the polished GCEs were equilibrated in 0.5 M H_2SO_4 solution by cycling between −0.5 and +1.4 V until steady-state voltammograms were achieved. Finally, the electrodes were rinsed with doubly distilled water and dried in air. Electrodes were polished in this way before each experiment, unless stated otherwise, and will be referred to as "conventionally polished" electrodes. Melamine was accumulated on the conventionally polished electrodes by cyclic voltammetric methods, and then the melamine-modified GCE (Mel-GCE) was rigorously washed with ethanol and water and sonicated for about 1 min in doubly distilled water to remove any physically adsorbed melamine. The effects of various parameters, such as the upper potential from 1.5 to 2.0 V and the pH of the accumulation solution, were optimized to obtain the best conditions for accumulation of melamine.

Horseradish Peroxidase Immobilization. HRP was covalently attached to Mel-GCE by glutaraldehyde (GA) as described previously.[40] After dipping in 1.0 mL of 1% GA solution for about 30 min, the Mel-GCE was taken out and washed thoroughly with water. The obtained electrode is denoted as GA-Mel-GCE. Then GA-Mel-GCE was immersed into 1 mL of HRP (4 mg/mL) solution and allowed to react for 30 min at 4 °C. The loosely bound HRP molecules were removed by sonication in distilled water for about 30 s. The electrode prepared by this process is called HRP-GA-Mel-GCE. In a control experiment, the same procedures were performed as described above except for the melamine accumulation process, and the obtained electrode is called HRP-GA-GCE. Atomic force microscopy (AFM) was used to characterize the surface of bare GCE, Mel-GCE, GA-Mel-GCE, and HRP-GA-Mel-GCE. All images were collected on a multimode Nanoscope IIIa scanning probe microscope (Veeco Co.) in the tapping mode. A microfabricated silicon cantilever with a bending spring constant of 20−80 N·m^{-1} and a resonance frequency of 229−287 kHz was used to collect the images at a scan rate of 1.0 Hz in air.

Colorimetric Assay Procedure. HRP-GA-Mel-GCE or HRP-GA-GCE was dipped into an assay solution containing guaiacol and H_2O_2 and incubated at 25 °C for a certain time. Then the assay solution was used for detection. All absorption spectra were measured with a Shimadzu (Kyoto, Japan) spectrophotometer. Each experiment was carried out at least in triplicate to make the experimental errors are lower than 5%. Influences such as the concentration of guaiacol and H_2O_2, pH of the reaction buffer, temperature, and incubation time were explored to obtain the best assay conditions for melamine detection.

Interference Study. For interference study, a series of interferents that may exist in infant milk powder or fish feed samples were studied. The interferents included some common inorganic ions such as Ca^{2+}, K^+, Na^+, Mg^{2+}, Zn^{2+}, Cl^-, NO_3^{2-}, amino acids such as methionine (Met), serine (Ser), valine (Val), isoleucine (Ile), leucine (Leu), threonine (Thr)and tryptophan (Trp), and vitamins B and C. They were added into pH 3.0 PBS solution for electrochemical accumulation. The accumulation processes were as described in the Electrochemical Accumulation of Melamine section in the absence of melamine. Then the interferent-accumulated electrodes were used for HRP assembly and colorimetric assay. The tolerance limits for the interfering species were established at those concentrations which caused absorbance no more than 5.0% compared with that of 1.0 × 10^{-8} M melamine.

Reproducibility Measurement. The reproducibility experiments were performed by using the same electrode seven times. After each experiment, the conventionally polished electrode was used for the accumulation of 5.0 × 10^{-9} M melamine, HRP assembly, and colorimetric assay.

Assay Validation. Liquid chromatography with electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS) was used to verify the application of the EA-ECA method. The LC-ESI/MS/MS experiment was performed by using an Accela LC system coupled with a triple-quadrupole mass spectrometer (Thermo Finnigan TSQ Quantum Access, San Jose, CA) via a reported procedure.[41] Chromatographic separation was achieved on Capcell CR 1: 4 (2.0 × 150 mm, S-5) at 40 °C. The mobile phase consisted of solvent A (acetonitrile) and solvent B (water containing 10 mM NH_4AC). The pH of the mobile phase was adjusted to 3.5. The flow rate was 0.20 mL·min^{-1}. The injection volume was 25 μL. The mass spectrometer was operated in the positive-ion electrospray ionization mode, and the detailed mass spectral conditions were as follows: positive-ion mode; capillary voltage 3.8 kV; desolvation temperature 350 °C; collision gas, helium.

RESULTS AND DISCUSSION

Electrochemical Behavior of Melamine on Electrodes. Cyclic voltammetry was used to examine the electrochemical behaviors of melamine on bare GCEs. The representative cyclic voltammograms of 1.0 × 10^{-8} and 1.0 × 10^{-4} M melamine at GCE in pH 3.0 solution are shown in Figure 1. No oxidation peak was observed during the positive potential scan from 0.0 to 2.0 V in the solution containing 1.0 × 10^{-8} M melamine. However, an increase in oxidation peak currents was observed at potentials more positive than 1.2 V (versus standard calomel electrode, SCE) (Figure 1a). When the GCE was cycled in a solution containing 1.0 × 10^{-4} M melamine, an anodic peak appeared at about 1.5 V during the first positive direct scan (Figure 1b). This peak was caused by the electro-oxidation of melamine. No reduction peak on the reverse scan was observed, indicating that the oxidation of melamine was irreversible. During the following cyclic sweeps, the anodic peak current decreased continuously. A steady state was obtained after 20 cycles. It might be caused by the accumulated melamine, which hindered the direct electron transfer and/or the diffusion of

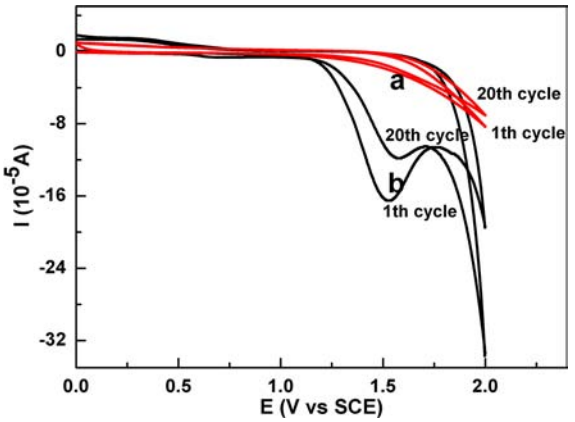


Figure 1. Cyclic voltammograms (CVs) of a glassy carbon electrode measured in the presence of (a) 1.0×10^{-8} M and (b) 1.0×10^{-4} M melamine in pH 3.0 solution.

melamine molecules to the electrode surface. The oxidation of melamine and its grafting on the GCE[30] are illustrated in Scheme 2. Melamine has been accumulated on the electrode through the meta-position of the amino group[42] by cyclic voltammetry. The remaining two unattacked amine groups are capable of conjugating HRP.

Characterization of Mel-GCE and HRP-Mel-GCE. AFM was used to monitor the construction process of the proposed sensor. A homogeneous structure of the bare GCE is shown in Figure 2A. Compared to the highly flat surface of the bare GCE, some clusters appeared on the electrode surface after the accumulation of melamine, as shown in Figure 2B. This indicated the successful accumulation of melamine on the electrode by cyclic voltammetry. The AFM image of Mel-GCE did not change after the covalent linkage of GA (Figure 2C). However, a rolling hill-like appearance, generally characteristic of any globular protein, was observed[43] after the assembly of HRP, as shown in Figure 2D. From these images we concluded that the sensor for the detection of melamine has been prepared.

Feasibility of the Assay Procedure. UV−visible spectroscopy has been used to test the feasibility of this method. The absorption spectrum of guaiacol oxidized by the HRP-GA-GCE is shown in Figure 3. Two absorption peaks were observed at wavelengths of 417 and 470 nm in this figure, indicating the formation of a color product. The color of the product was amber, as shown in the inset of Figure 3. To the naked eye, the presence of melamine could be directly observed through the color change of the assay solution, realizing the qualitative detection of melamine in a very convenient way.

To investigate whether melamine played an important role in the color change of the assay solution or not, HRP-GA-GCE was used for comparison. Without the melamine-accumulating process, no absorption was monitored in the control experiment (Figure 3A) and no color change was observed in the guaiacol/H_2O_2 system (inset, Figure 3). This experiment clearly indicated that the accumulated melamine was involved in the covalent binding of HRP. Thus, it could be indirectly determined via the assembled HRP.

Optimization of Accumulation of Melamine. Effects of various parameters, such as the upper potential limit from 0.0 to 2.0 V and the solution pH, on accumulation of melamine

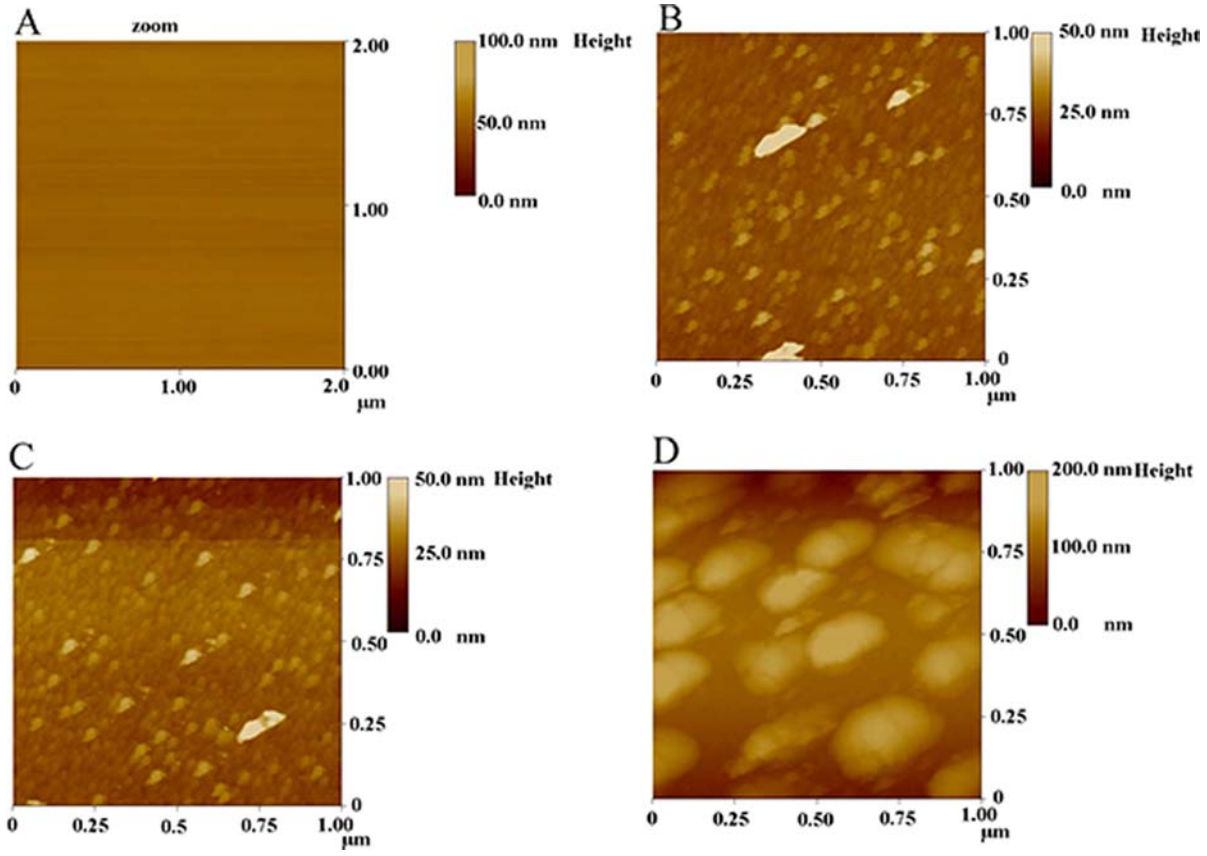


Figure 2. AFM images of (A) bare GCE, (B) Mel-GCE, (C) GA-Mel-GCE, and (D) HRP-GA-Mel-GCE surfaces.

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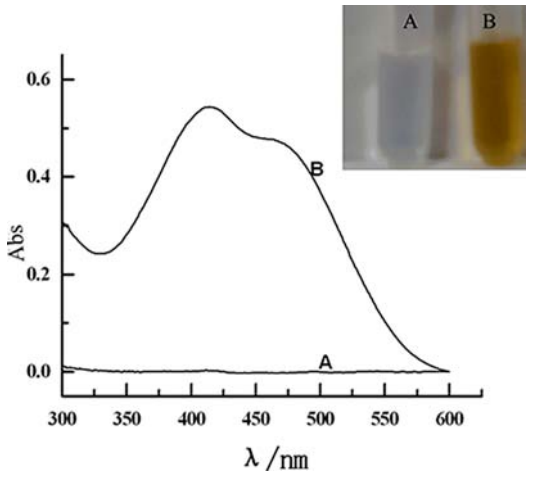

Figure 3. UV−vis spectra and (inset) photographs of oxidation product of guaiacol by (A) HRP-GA-GCE and (B) HRP-GA-Mel-GCE.

were studied. Cycling to potentials below 1.5 V would not cause the accumulation of melamine, indicating that the oxidation potential was very important. Therefore, we set the scan range between 0.0 and 2.0 V for the accumulation of melamine.

The effect of solution pH on the performance of the EA-ECA method is demonstrated in Figure 4A. It was investigated by checking the absorption intensity of the assay solution. When 1.0×10^{-8} M melamine was accumulated on the electrode in different pH solutions, the absorption intensity gradually increased from 0.0 to 3.0. This indicated that more melamine was accumulated on the electrode with increasing solution pH. In acid solutions, melamine would transfer into ammeline, ammelide, or cyanuric acid, thus causing the decrease of the signal. When the pH value was further increased from 3.0 to 7.0, the peak current gradually decreased. Because the highest signal was provided by a pH 3.0 PBS solution, the other parameters governing this method were studied and optimized at this pH.

Optimization of Colorimetric Assay Conditions. Guaiacol and H_2O_2 concentrations would affect the absorption of the assay solution. When 30 μL of 8.8 mM guaiacol, 100 μL of 0.04 M H_2O_2, and 2.9 mL of PBS were mixed together, we obtained the highest absorption intensity.

As shown in Figure 4B, the colorimetric assay catalyzed by the immobilized HRP is pH-dependent. The highest absorbance was reached when the pH of the assay solution was 7.0. Therefore, we propose 7.0 as the optimum pH for the colorimetric assay.

It is well-known that the activities of enzymes are highly sensitive to variations of temperature. Higher temperature would result in a significant drop in lifetime of enzymes and decrease the sensor sensitivity. However, raising the working temperature would increase the reaction rate. In our system, the analytical sensitivity was highest at 308 K, as was reported previously.[44] To ensure practical application for real samples, a universal environment, such as room temperature, was preferable. Thus, all experiments were performed at room temperature (298 ± 1 K) in this study.

Under the above conditions, we studied the effect of incubation time on the formation of the colored compound. The absorption intensity increased with the rise in incubation

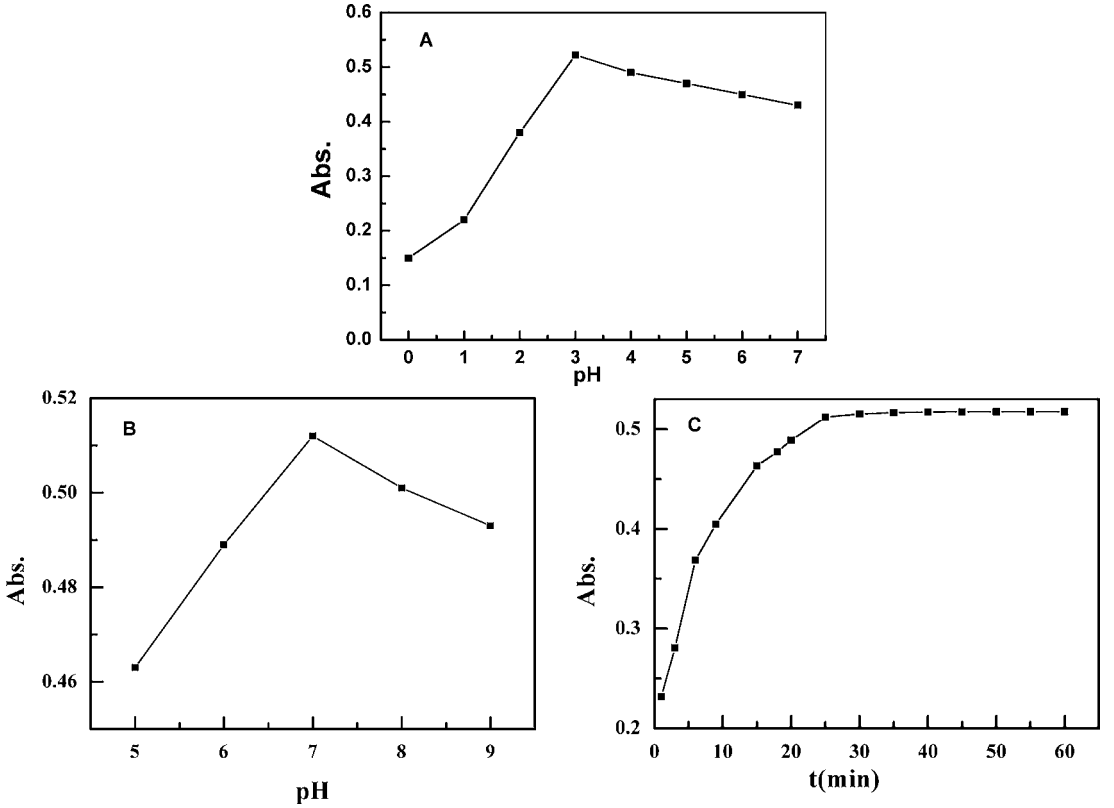

Figure 4. Influences of (A) pH of the accumulation solution, (B) pH of the assay solution, and (C) incubation time on absorption intensity of the assay solution.

time from 0 to 60 min, and equilibrium was achieved in 20 min at room temperature (Figure 4C). Thus, 20 min was finally selected for the detection of melamine.

Performance of the EA-ECA Method for Melamine Detection. Under the optimum conditions described above, a series of standard solutions of melamine were tested by the EA-ECA method. The relationship between melamine concentration and absorption intensity of the assay solution is shown in Figure 5. The absorption was enhanced with increasing

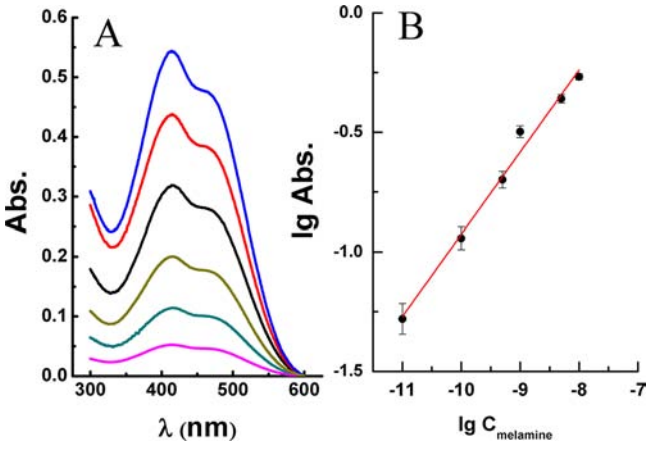

Figure 5. (A) UV−vis spectra of the assay solution with increasing amounts of melamine (from bottom to top). (B) Plot of logarithm of the absorption intensity at 417 nm against log [melamine] for melamine assay.

melamine concentration. Absorption at 417 nm was proportional to the logarithm of the melamine concentration over the range from 1.0×10^{-11} to 1.0×10^{-8} M (Figure 5B). An enzyme-catalyzed reaction is most likely to be zero-order initially, since substrate concentration is then highest.[45] When the reaction is zero-order, the absorption at 417 nm would be proportional to the melamine concentration. As substrate is used up, the enzyme's active sites are no longer saturated, substrate concentration becomes rate-limiting, and the reaction becomes first- or second-order. So with the increase in assembled HRP, the absorption at 417 nm is not proportional to melamine concentration any more. However, when both absorbance and concentration are transformed to logarithmic scales, the standard curve looks nearly linear. The regression equation of log Abs = −0.121 + 0.018 log C (molar) (R^2 = 0.989, n = 6) with a detection limit of 6.4×10^{-12} M (S/N = 3) is shown in Figure 5B. Different analytical methods have been applied for the detection of melamine, as listed in Table 1. Lou et al.[23] proposed a rapid and sensitive method for the determination of melamine by a surface-enhanced Raman scattering (SERS) strategy based on 4-mercaptopyridine (MPY) -modified gold nanoparticles (AuNPs). The detection limit was 0.1 parts per billion (ppb). Balabin and Smirnov[25] reported the detection of melamine by mid- and near-infrared (MIR/NIR) spectroscopy. This method was quick, sensitive (limit of detection, LOD, < 1 ppm), reliable, and robust. But it needed correct spectrum preprocessing (pretreatment) technique and a correct multivariate (MDA) algorithm for spectral analysis. Tsai et al.[21] used an oxidized polycrystalline gold electrode (poly GE) for the detection of adsorbed melamine by differential pulse voltammetry and impedimetric techniques. The linearity of this method was from 0.05 to 1.31 ppm. Compared with these reported methods, the EA-ECA method has the lowest detection limit and highest sensitivity. The improvement of the sensor is possibly caused by the synergetic effect of the electrochemical accumulation process and enzyme-induced amplification effect. The reproducibility of the sensor was evaluated, and the relative standard deviation was 4.5% (n = 7). For the intersensor repeatability of 10 sensors from the same batch, the relative standard deviation was 8.42% at a melamine concentration of 5.0×10^{-9} M. The interelectrode variability brought by variations during the electrochemical accumulation process could be one contributory factor. All the results indicated good reproducibility of the method.

Selectivity of the Assay. The interference studies showed that almost no absorbance of the assay solution was observed in the presence of 10 000-fold excesses of Ca^{2+}, K^+, Na^+, Mg^{2+}, Zn^{2+}, Cl^-, NO_3^{2-}, vitamin B, and vitamin C. Excesses (1000-fold) of methionine (Met), serine (Ser), valine (Val), isoleucine (Ile), leucine (Leu), and threonine (Thr) did not cause absorbance in the assay solution. Tryptophan (Trp) in 50-fold excess has no interference in the assay. Therefore, it is promising to apply this method to detect melamine in milk products and fish feed.

Real Sample Analysis and Validation of the Method. In order to evaluate the validity of the EA-ECA method, it was used to determine melamine in several infant formula powders and fish feed samples. Since no melamine residue was detected in those samples, they were spiked with melamine at different concentration levels to determine the recoveries (Table 2). It

Table 2. Recovery Tests of Melamine in Infant Milk Powders and Fish Feed Sample

sample	orig concn (M)	added ($\times 10^{-9}$ M)	found ($\times 10^{-9}$ M)	recovery (%)	RSD[a] (%)
Infant Milk Powder					
1	0.0	10.00	9.45	94.5	4.4
2	0.0	5.00	4.75	95.0	3.7
3	0.0	4.50	4.38	97.3	3.8
4	0.0	1.00	1.02	102.0	3.5
5	0.0	0.50	0.49	98.0	3.3
Fish Feed Samples					
6	0.0	5.00	4.83	96.6	2.9
7	0.0	8.00	8.23	102.8	4.5

[a]Relative standard derivation (RSD) was calculated from six recovery data values.

was found that the EA-ECA method demonstrated satisfactory recovery in the range of 94.5−102.8%, which was in good agreement with the amounts spiked. This result suggests that the EA-ECA method is applicable for the determination of melamine in real samples in the concentration range from 1.0×10^{-8} to 5.0×10^{-10} M.

LC-EIS/MS/MS method was used to test the applicability of the EA-ECA method. Both methods were used to analyze the same melamine-contaminated fish feed sample. The LC-ESI/MS/MS chromatogram of melamine external standard is shown in Figure 6a. The LC-ESI/MS/MS of one melamine-tainted fish feed sample is shown in Figure 6b. The concentration of melamine in the tainted sample is 2.4×10^{-7} M. At this concentration, the EA-ECA method could detect melamine qualitatively but not quantitatively. When we diluted the fish food feed sample 100 times, LC-EIS/MS/MS could not obtain the melamine concentration. However, the color of the assay

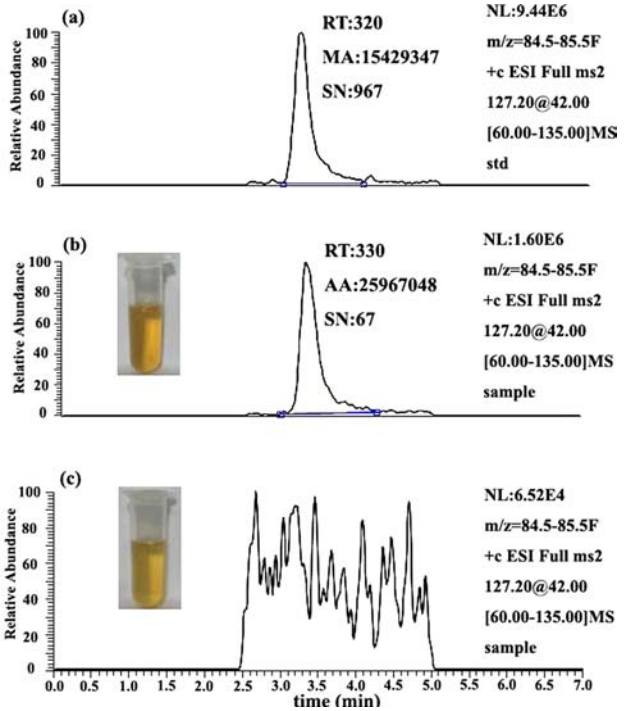

Figure 6. LC-ESI/MS/MS chromatograms of (a) melamine external standards, (b) fish feed sample tainted with 2.4×10^{-7} M melamine, and (c) fish feed sample tainted with 2.4×10^{-9} M melamine.

solution (Figure 6c) was still observed. The melamine concentration was 2.0×10^{-9} M in this sample, which was detected by the EC-ECA method. Therefore the EC-ECA method could be used for the detection of melamine at low concentrations. The result indicated the validity and sensitivity of the method for melamine detection.

In summary, a new method has been proposed for the detection of subnanomolar melamine, based on electrochemical accumulation coupled with enzyme colorimetric assay. This method is simple and sensitive. It has been successfully applied for the detection of melamine in infant milk powders and fish feed samples. The ease of performance, cost effectiveness of the system, and good results obtained in terms of recovery in real samples prove the potentiality of this assay as a screening method for melamine detection in milk powders and fish feed. Future work will be focused on the accumulation mechanism.

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Notes

The authors declare no competing financial interest.

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