DOI:10.1021/jf900132u

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Subpicogram Determination of Melamine in Milk Products Using a Luminol−Myoglobin Chemiluminescence System

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A sensitive chemiluminescence method based on a luminol−myoglobin system is proposed for the determination of melamine in milk products. It was found that the mixed solutions of melamine and myoglobin could react to form a complex on line, which could greatly inhibit the chemiluminescence intensity generated from the reaction between luminol and myoglobin. The decrease in chemiluminescence intensity was proportional to the concentration of melamine, giving a calibration graph linear over the concentration from 10 pg mL^{-1} to 50 ng mL^{-1} (R^2 = 0.9988) with a detection limit of 3 pg mL^{-1} (3σ). At a flow rate of 2.0 mL min^{-1}, one analysis cycle, including sampling and washing, could be accomplished in 20 s with a relative standard deviation of <4.0%. The proposed method was applied successfully to the determination of melamine in milk products, and the recovery was from 93.4 to 106.5%. The possible mechanism of luminol−myoglobin−melamine reaction is given.

KEYWORDS: Melamine; myoglobin; luminol; chemiluminescence; flow injection

INTRODUCTION

Melamine (2,4,6-triamino-*s*-triazine) is a nitrogen-containing compound used in the manufacture of plastics, in the production of melamine−formaldehyde resins for surface coatings, laminates, and adhesives, and in the production of flame retardants. Recently, pet foods contaminated with melamine led to renal disease and/or deaths in dogs and cats (in the spring of 2007, United States) (*1*−*3*), and the contamination of infant milk formula powder (Sanlu brand, etc.) with melamine caused many children to suffer renal complications (in the fall of 2008, China) (*4*−*6*), creating an urgent need for a sensitive, reliable, and rapid procedure for the determination of melamine in food.

HPLC with UV detection is currently the main method of quantitative determination of melamine (*7*−*10*). Commercial enzyme-linked immunosorbent assay (ELISA) technology used for the detection of melamine has been reported (*11*). HPLC-MS/MS has also been applied to detect melamine in food and kidney tissue (*12*−*14*). Chemiluminescence (CL) has attracted increasing attention in various fields owing to its high sensitivity, wide linear range, and simple instrumentation (*15*−*19*), but no CL procedure has been utilized for the determination of melamine to date.

It was previously reported that myoglobin (Mb), which contains a single iron protoporphyrin or heme moiety in the ferric state Mb(Fe^{III}), could react with luminol, yielding CL emission (*20*). It was also found that ligands such as fluoride, cyanide, and thiocyanate binding to the iron ion in the heme structure inhibit the CL reaction (*20*). In this work, it was found that melamine could bind to Mb, forming a complex, which decreased Mb concentration, resulting in inhibition of the CL intensity generated from the luminol−Mb reaction in alkaline medium. The decrement of CL intensity was proportional to the concentration of melamine, ranging from 0.01 to 50.0 ng mL^{-1} with the detection limit of 3 pg mL^{-1} (3σ). The proposed method was applied successfully to the determination of melamine in milk products, and the recovery was from 93.4 to 106.5%. This method offered the advantages of simplicity of apparatus, less reagent consumption, high sensitivity and sampling efficiency, wide linear range, and ease of handling.

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EXPERIMENTAL PROCEDURES

Reagents. All chemicals used were of analytical reagent grade. Water purified in a Milli-Q system (Millipore, Bedford, MA) was used throughout. Standard solution of melamine was supplied by the Xi'an Supervision and Inspection Institute of Product Quality. Luminol (Fluka, Biochemika) was obtained from Xi'an Medicine Purchasing and Supply Station, China. Horse heart Mb (Sigma) was used as received without further purification.

Stock solutions of melamine (1.10 mg mL^{-1}) was prepared in 20% methanol and stored at 4 °C. Working standard solutions of melamine were prepared daily from the above stock solution by appropriate dilution as required. Luminol (2.5×10^{-2} mol L^{-1}) was prepared by dissolving 0.44 g of luminol in 100 mL of 0.01 mol L^{-1} NaOH solution in a brown calibrated flask.

Apparatus. A peristaltic pump of the IFFM-E Luminescence Analyzer (Xi'an Remax Electronic Science-Tech. Co. Ltd., Xi'an, China) was applied to deliver all streams. PTFE tubing (1.0 mm i.d.) was used throughout the manifold for carrying the CL reagents. A

 Published on Web 4/1/2009

six-way valve with a loop of 100 μL was used for sampling. The flow cell was made by coiling 15 cm of colorless glass tube (1.0 mm i.d.) into a spiral disk shape with a diameter of 2 cm and placed close to the photomultiplier tube (PMT) (model IP28, Hamamatsu, Japan). Extreme precautions were taken to ensure that the sample compartment and PMT were light-tight. The CL signal produced in the flow cell was detected without wavelength discrimination, and the PMT output was recorded by PC with an IFFE-E client system (Remax, Xi'an, China). A schematic diagram of the flow injection (FI)−CL system used in this work was described in the literature (*21*).

Procedures. Flow lines were inserted into the sample, Mb, luminol, and carrier (pure water), respectively. The pump was started at a constant speed of 2.0 mL min^{-1} to wash the whole system until a stable baseline was recorded. Then 100 μL of luminol solution was injected into the carrier stream by injection valve, merged with the mixed solution stream of melamine and Mb. The mixed solution in an alkaline medium was delivered into the CL cell, producing CL emission, detected by the PMT and luminometer. The concentration of sample could be quantified on the basis of the decrement of CL intensity, $\Delta I = I_0 - I_s$, where I_s and I_0 are CL signals in the presence and in the absence of melamine, respectively.

Sample Preparation. About 2.00 g of milk products was weighed and placed into a 50 mL PTFE centrifuge tube, and then 15 mL of 1% trichloroacetic acid was added to promote protein precipitation. The mixture was dissolved ultrasonically for 20 min and then centrifuged at 4000 rpm for 10 min (*22*). Then the upper clear solution was filtered through a 0.45 μm membrane filter and diluted to the mark in a 25 mL calibrated flask. Suitable aliquot samples from this solution were taken for determination.

RESULTS AND DISCUSSION

CL Intensity−Time Profile of Melamine in Luminol−Mb System. Prior to the flow system being carried out, the kinetic curve was examined by dynamic method. The kinetic profile for CL intensity of the luminol−Mb reaction versus time was tested using 1.0×10^{-8} mol L^{-1} Mb and 2.5×10^{-5} mol L^{-1} luminol in 0.05 mol L^{-1} NaOH solution. As **Figure 1** shows, CL intensity reached a maximum at 4.5 s after the reagents were mixed and vanished within 18 s thereafter. It can be seen that the CL signal decreased from 310 to 195 in the presence of 1.0 ng mL^{-1} melamine.

Effect of pH of Mb. In an aqueous solution of Mb the following equilibrium exists: $Mb + O_2 \rightleftharpoons^{K_1} MbO_2$ and $K_1 = 9.2 \times 10^5$ mol L^{-1}, revealing that Mb occurs mainly in the MbO_2 state (*23*) and that MbO_2 is easily autoxidized to Mb (Fe^{III}) below pH 6.5, whereas above pH 8.0 MbO_2 with ferro-heme is dominant (*24*). The influence of Mb pH on CL was tested in the flow system. It was shown that the CL intensity varied with the pH of Mb solutions, obtaining the maximum CL signal at pH 6.5. Therefore, Mb solutions were prepared directly in water at pH 6.5.

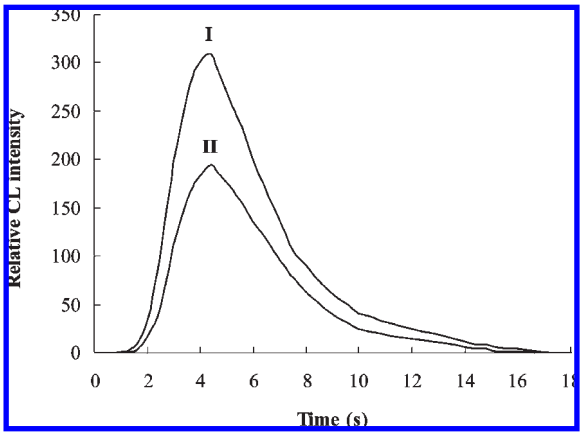

Figure 1. Kinetic CL intensity−time profile: I, CL intensity in the absence of melamine; II, CL intensity in the presence of melamine (1.0 ng mL^{-1})

Effect of Mb, Luminol, and NaOH Concentration. The effect of Mb and luminol concentration on the CL intensity was investigated over the ranges of 1.0×10^{-9} to 1.0×10^{-7} mol L^{-1} and 5.0×10^{-7} to 5.0×10^{-4} mol L^{-1}, respectively. It was found that the CL intensity reached maximum with 1.0×10^{-8} mol L^{-1} Mb and afforded approximately constant CL intensity over 1.0×10^{-8} mol L^{-1}, and the maximum CL intensity could be obtained when using a concentration of 2.5×10^{-5} mol L^{-1} luminol. Therefore, 1.0×10^{-8} mol L^{-1} Mb and 2.5×10^{-5} mol L^{-1} luminol were chosen as the optimum concentrations and used in subsequent experiments. Due to the nature of the luminol reaction, which is more favored under alkaline conditions, NaOH was introduced into the luminol solution to increase the sensitivity of the system. A series of NaOH solutions with different concentrations (0.005, 0.01, 0.03, 0.05, 0.1, and 0.2 mol L^{-1}, respectively) were tested. The CL intensity versus concentration of NaOH solution plot reached maximum at about 0.05 mol L^{-1}, and this concentration was employed in subsequent experiments.

Effect of Flow Rate and Length of Mixing Tubing. The effect of flow rate on CL intensity was examined in the range from 0.5 to 5 mL min^{-1}. It was found that the CL intensity increased with increase in flow rate, probably because this CL reaction was a fast process. As a compromise between reagent consumption and sensitivity, a 2.0 mL min^{-1} flow rate on each line was recommended. The length of the mixing tubing was also adjusted to yield maximum light emission in the cell. It was found that a 10.0 cm mixing tube afforded the best results with regard to sensitivity and reproducibility.

Performance of Proposed Method for Melamine Measurement. Under the optimum conditions described, a series of standard solutions of melamine were tested by the FI−CL system. The decrement of CL intensity was proportional to the logarithm of melamine concentration over the range from 0.01 to 50.0 ng mL^{-1}, given the regression equation $\Delta I_{CL} = 8.854 \ln C_{melamine} + 44.46$ ($R^2 = 0.9988$, $n = 7$), with a detection limit of 3 pg mL^{-1} (3σ). A complete analysis, including sampling and washing, could be performed in 20 s, yielding a throughput of 180 h^{-1} with relative standard deviations (RSDs) of 3.57, 1.67, and 1.05% for 0.01, 0.5, and 5 ng mL^{-1}, respectively.

Operational Stability of the FI−CL System. A 100 μL luminol alkaline solution was injected into the flow system merged with the mixed solution stream of melamine and Mb. The CL intensity ($\Delta I = I_0 - I_s$) of melamine concentrations of 0.1 and 1.0 ng mL^{-1} was recorded to test the stability of the system. The experiment lasted for 3 days, and the flow system was regularly used over 8 h per day. The results of these replicate experiments are listed in **Table 1**. Each result is the average of seven separate determinations, and the RSDs were < 4.0%. It was found that the system exerted very good stability.

Table 1. Stability Test of FI-CL System under Different Concentrations of Melamine[a]

time (days)	I_{CL}, blank	RSD (%)	I_{CL}, 0.1 ng mL^{-1}	RSD (%)	I_{CL}, 1.0 ng mL^{-1}	RSD (%)
1	285.0	1.19	259.4	2.67	241.6	2.12
2	284.8	2.86	259.0	3.52	242.8	1.78
3	284.4	1.65	258.8	2.24	241.0	2.82

[a] Average of seven determinations.

Table 2. Results of Determination of Melamine in Spiked Milk Powder and Liquid Milk Samples[a]

sample[b]	added (ng mL^{-1})	found (ng mL^{-1})	RSD (%)	recovery (%)	by the proposed CL method/spiked (μg g^{-1})
1-1	0	0.19	1.52	107.9	0.47/0.50
	0.07	0.27	1.84		
1-2	0	0.20	1.02	104.7	0.51/0.50
	0.10	0.31	1.87		
1-3	0	0.18	0.79	97.0	0.45/0.50
	0.20	0.37	0.86		
1-4	0	0.19	2.28	108.6	0.48/0.50
	0.30	0.51	1.31		
1-5	0	0.21	1.94	92.6	0.53/0.50
	0.40	0.59	2.31		
2-1	0	0.40	1.19	96.8	1.01/1.00
	0.20	0.59	1.58		
2-2	0	0.41	1.16	103.5	1.03/1.00
	0.30	0.72	1.09		
2-3	0	0.39	1.91	101.6	0.98/1.00
	0.40	0.80	1.44		
2-4	0	0.41	1.77	105.3	1.03/1.00
	0.50	0.94	1.39		
2-5	0	0.41	2.07	96.2	1.03/1.00
	0.60	0.99	2.30		

[a] Average of seven determinations. [b] Samples 1-1 to 1-5, Yinqiao liquid milk. Samples 2-1 to 2-5, Quechao milk powder.

Table 3. Results of Determination of Melamine in Spiked Yogurt Samples[a]

sample[b]	added (ng mL^{-1})	found (ng mL^{-1})	RSD (%)	recovery (%)	by the proposed CL method/spiked (μg g^{-1})
1-1	0	0.46	1.62	93.9	1.15/1.25
	0.30	0.74	1.73		
1-2	0	0.48	0.55	98.8	1.20/1.25
	0.50	0.97	1.94		
1-3	0	0.51	1.70	90.8	1.28/1.25
	0.70	1.14	1.02		
1-4	0	0.67	0.71	96.2	1.68/1.75
	0.50	1.15	0.95		
1-5	0	0.66	0.97	104.2	1.65/1.75
	0.70	1.39	1.33		
2-1	0	0.83	1.06	104.4	2.08/2.00
	0.50	1.36	1.52		
2-2	0	0.82	1.46	94.5	2.05/2.00
	1.00	1.77	1.74		
2-3	0	0.99	1.03	95.2	2.48/2.50
	0.70	1.66	0.81		
2-4	0	0.97	2.55	108.0	2.43/2.50
	1.00	2.05	1.28		
2-5	0	1.02	2.83	97.7	2.55/2.50
	3.00	3.96	1.05		

[a] Average of seven determinations. [b] Samples 1-1 to 1-5, Yili yogurt. Samples 2-1 to 2-5, Yinqiao yogurt.

Table 4. Results of Determination of Melamine in Contaminated Milk Powder[a]

sample	added (ng mL^{-1})	found (ng mL^{-1})	RSD (%)	recovery (%)	amount of melamine in sample (μg g^{-1}) by CL method	by HPLC[b]
1-1	0	1.01	1.08	94.7	2.53	
	0.50	1.48	1.29			
1-2	0	0.98	1.77	96.8	2.45	
	0.70	1.66	0.65			
1-3	0	1.02	1.49	96.2	2.55	1.50
	1.00	1.98	1.68			
1-4	0	1.02	1.18	101.4	2.55	
	2.00	3.05	1.35			
1-5	0	1.01	1.95	102.2	2.53	
	3.00	4.08	1.14			
2-1	0	2.06	3.75	98.3	5.15	
	0.70	2.75	3.50			
2-2	0	2.01	1.65	93.4	5.03	
	1.00	2.94	2.11			
2-3	0	1.92	1.76	100.8	4.80	4.50
	2.00	3.93	1.96			
2-4	0	1.95	1.86	106.5	4.88	
	3.00	5.15	1.31			
2-5	0	1.96	1.77	97.2	4.90	
	4.00	5.86	2.31			

[a] Average of seven determinations. [b] Data (batches 20080806 H0102H and 20080827) promulgated by General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (*26*).

Interference Studies. The interference of foreign species was tested by analyzing a standard solution of melamine (0.1 ng mL^{-1}) into which increasing amounts of potential interfering substances were added. The tolerable concentrations of foreign species with respect to 0.1 ng mL^{-1} melamine for interference at the 5.0% level were less than 1.0 μg mL^{-1} for NO_3^-, Ac^-, I^-, SO_4^{2-}, PO_4^{3-}, BrO_3^-, glucose, fructose, lactose, borate, oxalate, and malic acid; 0.5 μg mL^{-1} for NH_4^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, methanol, ethanol, VB_1, VB_2, and VB_6; 0.3 μg mL^{-1} for urea; 0.1 μg mL^{-1} for lysozyme, uric acid, and trichloroacetic acid; and 10 ng mL^{-1} for Cu^{2+}, Zn^{2+}, Ni^{2+}, Cr^{3+}, Fe^{2+}/Fe^{3+}, and vitamin C, respectively. It should be noted that the interference of some species often found in milk was studied. The content of Ca^{2+}, urea, vitamin B, and vitamin C in milk was, respectively, about 370, 300, 200, and 20 mg kg^{-1} (*25*), which showed no interference in the determination of melamine after dilution by (1.0×10^4)-fold with water.

APPLICATIONS

Determination of Melamine in Spiked Milk Powder, Liquid Milk, and Yogurt Samples. To validate the proposed method to detect melamine specifically, known quantities of melamine were spiked into the samples of milk powder, liquid milk, and yogurt and determined. The samples of Quechao milk powder, Yili yogurt, Yinqiao liquid milk, and yogurt were purchased from the local market and prepared as described under Sample Preparation. The contents of melamine in the spiked samples were quantified according to the standard addition method, and the results are listed in **Tables 2** and **3**, with recoveries ranging from 90.8 to 108.6% and RSDs of $< 3.0\%$.

Determination of Melamine in Contaminated Milk Powder. The proposed method was applied to the determination of melamine in two kinds of Sanlu milk powder purchased from the local market, which have been confirmed to contain melamine (batches 20080806 H0102H and 20080827). The samples were prepared as described under Sample Preparation, which were also determined by the standard addition method, and the results are listed in **Table 4**. The recoveries ranged from 94.7 to 102.2% and from 93.4 to 106.5%, with RSDs of $< 4.0\%$. The results of HPLC (the same batches, 20080806 H0102H and 20080827) are also listed, which were promulgated by General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (*26*).

Possible Mechanism of the CL Reaction. It was reported that the binding of ligand to Mb could significantly increase or inhibit luminol−Mb CL reaction. The procedures of determination of nitrite, formaldehyde, gatiloxacin, and clindamycin were established, and the possible interaction mechanisms were given by our group (*27*−*30*). In this work, the possible mechanism of luminol−Mb−melamine reaction was investigated using the UV and CL method, and the results are shown in **Figure 2** and **Table 5**. **Figure 2** shows the UV absorption spectra profile of different reaction types including Mb, Mb−melamine, melamine, luminol, and luminol−melamine. It was clear that the absorption spectra of luminol (curve IV) and luminol−melamine (curve V) had no obvious change, indicating that there was no reaction

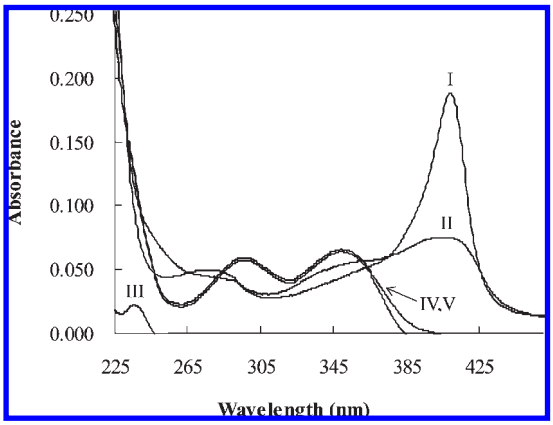

Figure 2. UV absorption spectra profile of different reaction types: I, Mb; II, Mb−melamine; III, melamine; IV, luminol; V, luminol−melamine. The concentrations of Mb, melamine, and luminol were 1.0×10^{-6}, 1.0×10^{-6}, and 1.0×10^{-5} mol L^{-1}, respectively.

Table 5. Results of Different Reaction Types by UV[a] and Dynamic CL[b]

type of reaction	A_{409nm}	I_{CL}
Mb	0.1881	
Mb−melamine	0.0752	
luminol−Mb		310
luminol−Mb−melamine		195

[a] The concentrations of Mb, melamine, and luminol were 1.0×10^{-6}, 1.0×10^{-6}, and 1.0×10^{-5} mol L^{-1}, respectively. [b] The concentrations of Mb, melamine, and luminol were 1.0×10^{-8} mol L^{-1}, 1.0 ng mL^{-1}, and 2.5×10^{-5} mol L^{-1}, respectively.

Table 6. Comparison of Different Methods for Determination of Melamine

method	LOD (ng mL^{-1})	recovery (%)	sample	ref
HPLC- UV	100	71−105	pet food	*9*
	20	98.7−101.1		*10*
	10	86−89	chard	*12*
HPLC-MS/MS	3	63.8	fish	*13*
		86	kidney tissue	*14*
ELISA	9	78−82	dog food	*11*
proposed CL	0.003	93.4−106.5	milk powder	
		97.0−108.6	liquid milk	

between luminol and melamine. The absorptions of Mb (Fe^{III}) and Mb(Fe^{III})−melamine were obtained by locating λ_{max} at 409 nm, which was the maximum absorption of the characteristic wavelength for Mb(Fe^{III}) (*31*), and the results are listed in **Table 5**. The absorption value of Mb(Fe^{III}) at λ_{max} at 409 nm decreased from 0.1881 to 0.0752, and the CL signal of the luminol−Mb system reduced from 310 to 195 in the presence of melamine. Because the luminol−Mb CL emission could be inhibited significantly in the presence of some ligands (*20*), it was supposed that melamine could bind to Mb, forming a complex, which decreased Mb concentration and resulted in the inhibition of the CL signal from luminol−Mb reaction system.

The calibration curve experiment using the standard addition method in Yinqiao liquid milk samples was carried out, and a good linear equation, $\Delta I_{CL} = 35.157 \ln C_{melamine} + 70.94$, $R^2 = 0.9916$, was also obtained. Comparison between the presented FI−CL procedure and reported methods for the determination of melamine is summarized in **Table 6**. The proposed method had the highest sensitivity with a LOD of 0.003 ng mL^{-1} and good recovery compared with the existing methods.

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Received for Review January 13, 2009. Revised manuscript received March 14, 2009. Accepted March 15, 2009. We gratefully acknowledge financial support from Shaanxi Province Nature Science Foundation, the Foundation of Ministry of Education, the NWU Graduate Innovation and Creativity Funds, China, Grants 2006B05, 07JK395, and 08YZZ42, and the Foundation of Shaanxi Key Laboratory of Analytical Chemistry.