Room-temperature phosphorescence determination of melamine in dairy products using L-cysteine-capped Mn-doped zinc sulfide (ZnS) quantum dots

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ABSTRACT

A simple, sensitive, and precise room-temperature phosphorescence method was developed for the determination of melamine in dairy products using L-cysteine-capped Mn-doped zinc sulfide (ZnS) quantum dots as a probe. This method is based on the quenching of the phosphorescence signal of quantum dots by the interaction with melamine. Under optimum conditions, phosphorescence intensity was quenched by various concentrations of melamine in a linear range from 50 to 500 ng/mL, with a detection limit of 5.95 ng/mL in 10 mM phosphate buffer (pH 7.4). The relative standard deviation for 5 replicate measurements was 0.15%. The developed method was applied to dairy products to determine melamine concentrations; recovery values ranged from 96.3 to 104.7%.

Key words: room-temperature phosphorescence, milk product, melamine, quantum dot

INTRODUCTION

Melamine (MEL; 1,3,5-triazine-2,4,6-triamine) is a trimer of cyanamide and is a nitrogen-rich chemical (Figure 1). The combination of MEL with triazines and cyanuric acid causes insoluble crystals to form in the kidneys, resulting in renal failure and bladder cancer (Vara Messler et al., 2012; Chansuvarn et al., 2013; Chang et al., 2014). Melamine is widely used in the manufacturing of plates, kitchenware, plastic products, paper, and glues. Although MEL may be used in the manufacturing of packaging for food products, it is not approved for direct addition to human food or animal feeds. Because of its high nitrogen level, MEL increases the protein level, but it cannot be detected by the Kjeldahl method. Melamine is inexpensive and easily accessible and thus is illegally added to protein-rich foods such as milk products to enhance the protein content (Vara Messler et al., 2012; Cao et al., 2013). In

many countries, the allowed limits of MEL are 1 ppm for infant formulas and 2.5 ppm for other food (Wang et al., 2014). According to the Turkish Food Codex (TFC), the levels of melamine should not exceed 1 mg/kg in powdered infant formula and 2.5 mg/kg in food (other than infant formula; TFC, 2012). Therefore, a simple and sensitive method is needed for the detection of MEL in dairy products.

Determination of MEL concentrations in protein-based food products such as milk products is important to prevent or detect adulteration. To date, several methods have been used for determination of MEL in milk products, including HPLC with detection systems such as UV (Yan et al., 2012; Beltran-Martinavarro et al., 2014), fluorescence (Finete et al., 2014; Zhang et al., 2014), mass spectrometry (Duan et al., 2011; Hassani et al., 2013; Khedr, 2013; Shin et al., 2014), capillary electrophoresis (Vallejo-Cordoba and Aaron, 2010; Katou and Wu, 2012; Vachirapatama and Maitresorasun, 2013; Wu and Yu, 2013; Himmelsbach and Thanh Vo, 2014; Kong et al., 2014), GC-MS (Koh et al., 2011; Jurado-Sanchez and Ballesteros, 2011; Pan et al., 2013; Wong and Mok, 2013), electrochemical methods (Araujo and Paixao, 2014; Guo et al., 2014; Xu et al., 2014; Xue et al., 2014), and spectrophotometry (Huang et al., 2010; Chansuvarn et al., 2013; Li et al., 2013; Liu et al., 2014). However, many of these methods are not suitable for routine analysis because of their complicated, time-consuming, and high-cost features. In particular, spectrophotometric methods require a derivatization step. Therefore, simple, sensitive, fast, and low-cost methods need to be developed.

Since their development, fluorescent quantum dots (QD) have increasingly replaced traditional organic fluorophores. Quantum dots are semiconductor nanoparticles with some advantages over organic and inorganic fluorophores: good optical properties, stability against photobleaching and chemical reaction, size-control luminescence, broad excitation bands and sharp emission bands, as well as high photoluminescence efficiency (Ren and Yan, 2012). Because of these properties, QD are widely used as fluorescent probes for analysis of various analytes. Typically, fluorescent QD

Received December 10, 2014.

Accepted January 30, 2015.

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are constructed from inorganic semiconductor core materials such as cadmium telluride (CdTe) or cadmium selenium (CdSe). Fluorescent QD have also been used in determination of MEL. These methods are based on fluorescence resonance energy transfer between MEL and different QD such as CdTe (Tang et al., 2013) and cadmium sulfide (CdS; Wang et al., 2012) or an inner filter effect of Au and Ag nanoparticles on the fluorescence of CdS (Cao et al., 2013), CdTe (Zhang et al., 2012), and ZnSe (Cao et al., 2014).

In this study, a room-temperature phosphorescence (RTP) method was developed for selective detection of MEL. Compared with fluorescence techniques, RTP is more selective and sensitive, and it has a longer emission lifetime and wider Stokes shift (Matsuoka and Yoshimura, 2010). Therefore, L-cysteine-capped Mn-doped zinc sulfide (ZnS) QD were synthesized and characterized. The use of different dopants can strongly modify the optical, electrical, and magnetic character of QD. Compared with traditional QD such as CdTe or CdSe, ZnS is an attractive semiconductor for doping to produce new types of quantum dots; Mn^{2+} and Zn^{2+} have equal electric charge and similar ionic radii, so Mn^{2+} can be easily incorporated into the ZnS crystal lattice without producing a great effect. Thus, ZnS is a more attractive host nanoparticle for doping to form QD with unique phosphorescence properties. Manganese-doped ZnS QD exhibit phosphorescence emission when excited at 290 nm. The working mechanism of these probes is based on quenching of their phosphorescence intensities. This type of QD has been used in the phosphorescent sensing of drugs and biomolecules without the need for any sample pretreatment.

The aim of this study was to synthesize L-cysteine-capped Mn-doped ZnS QD and use them as the probe to detect MEL. Melamine quenches the phosphorescence signal of Mn-doped ZnS QD. Based on this procedure, we propose a new method for MEL detection and quantification. The method allows the sensitive, selective, and rapid detection of MEL in dairy products.

MATERIALS AND METHODS

Materials and Chemicals

L-Cysteine, ZnSO_4 , MnCl_2 , and Na_2S (Merck, Darmstadt, Germany) were used for the preparation of Mn-doped ZnS QD. Melamine was obtained from Acros Organics (Geel, Belgium). Trichloroacetic acid ($\text{C}_2\text{HCl}_3\text{O}_2$) was obtained from Merck. A stock solution of 500 $\mu\text{g/mL}$ MEL was prepared in deionized water and stored at 5°C. Solutions of different concentrations of MEL were prepared by dilutions in buffer solution. All reagents were of analytical grade. Phosphate buf-

Figure 1. Structural formula of melamine.

fer (0.01 M, pH 7.4) was prepared in deionized water and the pH was adjusted using 5 M sodium hydroxide. Buffer solution and sodium hydroxide (1 and 5 M) were prepared using analytical-grade reagents and deionized water. Deionized water (18.2 M Ω -cm, Simplicity, Milli-Q water purification system; Millipore, Billerica, MA) was used for the preparation of aqueous solutions. In this study, commercial milk products (yogurt, cream, white cheese, coffee creamer, and follow-on milk) were collected from markets in Ankara Province, Turkey.

Apparatus

The phosphorescence measurements were performed with a Varian Cary Eclipse spectrofluorimeter with a 10- × 10-mm quartz cuvette (Varian, Palo Alto, CA). Excitation wavelength and slit widths were 290 and 10 nm, respectively. A xenon flash lamp was used as the light source.

Ultraviolet-visible spectrometric measurements were carried out using Shimadzu 160 A spectrometer (Shimadzu, Tokyo, Japan). The measurements were done using a pair of 10 × 10-mm-path-length quartz cells. The pH measurements were performed using a combined pH electrode with an Orion model 720 A pH meter (Thermo Orion, Beverly, MA). An MSE Mistral1000 type centrifuge (Loughborough, UK), J. P. Selecta type sonicator (J. P. Selecta, Barcelona, Spain), and vortex (Firlabo, Lyon, France) were used in sample preparation throughout the studies. Samples and standards were filtered using a 0.45- μm filter (Sartorius, Goettingen, Germany). All experiments were done at room temperature.

Synthesis of Mn-Doped ZnS QD

Synthesis of the Mn-doped ZnS QD was carried out in aqueous solution based on a published method with a minor modification (Wang et al., 2009). In this ex-

periment, 50 mL of 0.02 *M* L-cysteine, 5 mL of 0.1 *M* ZnSO₄, and 1.5 mL of 0.01 *M* MnCl₂ were added to a flask and mixed. Then, the pH of the mixture was adjusted to 11 with 1 *M* NaOH. After stirring, argon gas was passed through the flask contents at room temperature for 30 min to remove air, and 5 mL of 0.1 *M* Na₂S was added quickly to the solution to allow nucleation of the nanoparticles. The mixture was stirred for 20 min, and then the solution was aged at 50°C in the open air for 2 h to form L-cysteine-capped Mn-doped ZnS QD.

Sample Preparation

Two milliliters of milk sample or 1 g of dairy product such as yogurt, cream, cheese, or coffee creamer was transferred to centrifuge tubes, and 15 mL of 1% TCA was added. This mixture was vortexed thoroughly, placed in an ultrasonic bath for 20 min, and then centrifuged at $2,400 \times g$ for 15 min at room temperature. The supernatant was transferred to a 25-mL flask and filtered through a 0.45- μ m filter, and the pH was then adjusted to 7.4 (Gong et al., 2013).

Phosphorescence Experiments

Phosphorescence measurements were carried out at an excitation wavelength of 290 nm in the absence and presence of MEL. One hundred microliters of QD was diluted with 10 mM phosphate buffer solution (pH 7.4), and different volumes of MEL solution were added to detect the phosphorescence-quenching effect. Phosphorimetric measurements were done 2 min after the reaction was initiated.

RESULTS AND DISCUSSION

Characterization of the QD

L-Cysteine-capped Mn-doped ZnS QD were synthesized based on the reaction of zinc sulfate, manganese chloride, and sodium sulfide in aqueous solution, as described in the Synthesis of Mn-Doped ZnS QD section. Morphologies of QD have been characterized by He et al. (2008); QD have a spherical shape and almost uniform size (diameter of about 3.5 nm; He et al., 2008). The diameter of Mn-doped ZnS QD was calculated by using equation [1] (Fortes et al., 2011)

$$D = 9.8127 \times 10^{-7} \lambda^3 - 1.7147 \times 10^{-3} \lambda^2 + 1.0064 \lambda - 194.84, \quad [1]$$

where *D* is the diameter of the QD and λ is the wavelength of emission maximum. The calculated diameter of the Mn-doped ZnS QD prepared in this study was 3.5 nm.

The QD showed a broad UV absorption band between 200 and 300 nm, with 2 maxima around 209 and 290 nm (Figure 2a). Phosphorescence of L-cysteine-capped Mn-doped ZnS QD was not observed without the aging step, but QD exhibited a phosphorescence emission peak at 590 nm when excited at 290 nm after aging at 50°C under open air for 2 h (Figure 2b). This emission band is pH-dependent and stable in the range of pH 7.0 to 8.5 (He et al., 2008). The prepared QD were stable in water for at least 5 mo in the dark at 4°C, without notable precipitation, and the phosphorescence signal of QD was stable during this period (data not shown).

Optimization of pH

The phosphorescence intensity of the Mn-doped ZnS QD was pH dependent. As shown in Figure 3a, in acidic solution, the phosphorescence intensity of Mn-doped ZnS QD was low. The phosphorescence intensity increased steadily up to pH 7.4, was stable in the range of pH 7.4 to 8.0, and then decreased sharply from pH 8.0 to 9 (Figure 3a). The quenched phosphorescence intensity also changed with pH: it was stable between pH 7.4 and 8.0 and then decreased sharply (Figure 3b). Thus, a pH of 7.4 was selected for this method.

Reaction Time

We investigated the effect of reaction time on the phosphorescence intensity of Mn-doped ZnS QD in pH 7.4 phosphate buffer. The RTP intensity of QD was quenched quickly upon addition of MEL and the reaction reached equilibrium within 2 min, after which time the signal was stable. Therefore, the QD-MEL solutions were analyzed after 2 min (Figure 4).

Interferences

One objective of this study was to apply the developed method to determine the presence of MEL in dairy products. It is known that ions from salts in the samples can affect the RTP signal. Therefore, effects of many common ions such as Ca²⁺, Mg²⁺, K⁺, Na⁺ were examined. The RTP intensities of L-cysteine-capped Mn-doped ZnS QD and QD-MEL system were not affected by a 500-fold concentrations of K⁺, Na⁺, Ca²⁺, and Mg²⁺. No significant change in signal was observed. Thus, the developed method can be used for the analy-

Figure 2. The absorption spectrum (a) and phosphorescence spectrum (b) of Mn-doped zinc sulfide (ZnS) quantum dots.

sis of MEL in dairy products without potential interferences.

Validation of the Method

Linearity. To determine MEL in dairy product, we investigated the effect of MEL on RTP intensity of QD. The RTP intensity of Mn-doped ZnS QD was sensitive

to MEL. Measurements of the phosphorescence spectra were obtained in 10 mM phosphate buffer at pH 7.4. As shown in Figure 5, the quenching of RTP intensity of QD was dependent on the concentration of MEL. We detected a linear relationship between the quenched RTP intensity (ΔP) and the concentration of MEL in the range of 50 to 500 ng/mL, with a correlation coefficient of 0.995. The linear regression was $\Delta P = 0.708C$

Figure 3. Effect of the pH on the room-temperature phosphorescence (RTP) intensity of Mn-doped zinc sulfide (ZnS) quantum dots (a) and the quenched RTP intensity of quantum dots with melamine (b).

+ 44.06, where C was the concentration of MEL (ng/mL). The analytical data for the calibration graph are listed in Table 1.

Precision. System repeatability was determined by 5 replicate applications and measurement of phosphorescence intensity. Method repeatability was obtained from relative standard deviation (%) values obtained by repeating the assay 5 times on the same day (intra-day precision). Inter-day precision was assessed by assaying samples on 3 different days. The results of intra- and inter-day precision studies are shown in Table 1.

Limits of Detection and Quantification. Several approaches are given in the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guideline (ICH, 2014) to determine limit of detection (LOD) and limit of quantification (LOQ). In this study, the LOD and LOQ were calculated from the equations of $LOD = 3s/m$ and $LOQ = 10s/m$ (Swartz and Krull, 1997), where s is the standard deviation for 5 replicates and m is the slope of the calibration curve. The LOD and LOQ values were calculated as 5.95 and 19.8 ng/mL,

Figure 4. Effect of the reaction time on the room-temperature phosphorescence (RTP) intensity of Mn-doped zinc sulfide (ZnS) quantum dots (0 s is RTP signal of quantum dots only).

respectively (Table 1). Under optimal conditions, this LOD value is low enough for the determination of MEL in milk products; the maximum MEL concentrations allowed by TFC are 2.5 and 1 mg/kg for food (other than infant formula) and powdered infant formula, respectively.

Recovery. To test the accuracy and reproducibility of the proposed method, recovery studies were performed by spiking samples with an appropriate amount of the stock solution of MEL. The recovery values were calculated using the related regression equation after 3 measurements. The recovery values were in an acceptable range of 96 to 104% (Table 2).

Sample Analysis

Under optimal conditions, the proposed method was used to detect MEL in milk products, including follow-on milk, cheese, cream, coffee creamer, and yogurt. No interfering peaks were observed from any of the ingredients of the assayed samples (Table 2). As seen in the table, the detected values were below the acceptable

Table 1. Statistical evaluation of calibration data for quantitative determination of melamine

Item	Value
Linearity range (ng/mL)	50–500
Slope	0.71
Intercept	44.06
Correlation coefficient	0.995
SE of slope	0.11
SE of intercept	3.20
Limit of detection (ng/mL)	5.95
Limit of quantification (ng/mL)	19.83
Inter-day precision (3 d, n = 5; RSD %) ¹	0.15
Intra-day precision (n = 5; RSD %) ¹	0.49

¹RSD = relative standard deviation.

Figure 5. Effect of melamine concentration on the room-temperature phosphorescence (RTP) intensity of Mn-doped zinc sulfide (ZnS) quantum dots. The concentrations of melamine were (a) 0, (b) 50, (c) 100, (d) 150, (e) 200, (f) 250, (g) 300, (h) 350, (i) 400, (j) 450, and (k) 500 ng/mL. Delta P (ΔP) is the quenched RTP intensity, which is the difference between phosphorescence intensity of QD before and after addition of MEL. Inset figure (ΔP) shows quenched RTP intensity.

values. The literature contains a few reports on MEL detection using different methods, as summarized in Table 3. Our proposed method had good sensitivity, a wide linear range, a low RSD, and high recovery percentage compared with other techniques.

Response Mechanism

Quenching of phosphorescence signal refers to the decrease of phosphorescence intensity of a phosphorescent molecule due to molecular interactions. The

Table 2. Results of samples and recovery analysis of melamine (MEL) in different dairy products

Sample	MEL level	Added (ng/mL)	Recovered (ng/mL)	RSD ¹ (%)	Recovery (%)
Coffee creamer (ng/g)	866	50.0	50.0	0.22	100
		100.0	103.9	0.86	103.9
		150.0	152.3	0.87	101.5
White cheese (ng/g)	<DL ²	50.0	48.1	0.35	96.3
		100.0	104.7	0.34	104.7
		150.0	151.0	0.45	100.6
Yogurt (ng/g)	1,275	50.0	50.1	0.80	100.3
		100.0	104.0	0.91	104.0
		150.0	151.4	0.46	101.1
Cream (ng/g)	1,531	50.0	50.2	0.39	100.4
		100.0	103.4	0.48	103.4
		150.0	150.7	0.86	100.5
Follow-on milk (ng/mL)	<DL	50.0	48.8	0.89	97.6
		100.0	103.8	0.38	103.8
		150.0	150.7	0.85	100.4

¹Relative standard deviation.

²<DL = below detection limit.

Figure 6. Stern-Volmer plot for the phosphorescence quenching effect of melamine on Mn-doped zinc sulfide (ZnS) quantum dots (concentration of melamine ranged from 0.4 to 4 nM in 10 mM phosphate buffer, pH 7.4); P_0 and P are the phosphorescence intensities of quantum dots in the absence and presence of quencher, respectively.

mechanism is generally 1 of 2 types: dynamic or static quenching (Lakowicz, 1999). In dynamic quenching, the phosphorescent molecule and quencher contact when the molecules are at the excited state and the phosphorescent molecule returns to the ground state without emission. In static quenching, however, the phosphorescent molecule and quencher form a nonphosphorescent complex. To investigate the quenching mechanism, the phosphorescence quenching data from our study were analyzed using the Stern-Volmer equation (Eq. [2]; Lakowicz, 1999):

$$P_0/P = 1 + K_{app}[Q], \quad [2]$$

where P_0 and P are the phosphorescence intensities of QD in the absence and presence of quencher, respectively; K_{app} is the Stern-Volmer quenching constant; and $[Q]$ is the concentration of the quencher. The Stern-Volmer quenching constant, K_{app} , was determined by linear regression of a plot of P_0/P against $[Q]$.

The relationship between P_0/P and the increasing concentration of MEL presented a linear curve with a

correlation coefficient of $r = 0.9975$ in the range of 0.4 to 4 nM, which permits its use as a probe to determine MEL (Figure 6). The linear regression equation was $P_0/P = 0.1571[Q] + 0.9973$, where $[Q]$ is the concentration (nM) of MEL. According to this result, the RTP quenching mechanism was dynamic, and K_{app} was found to be $1.5 \times 10^5 M^{-1}$, indicating that MEL and QD interacted strongly. Melamine has 3 amino groups, which could strongly interact with the QD. Amino acids in samples also interact with QD via their amino groups, but these interactions are much weaker compared with the interaction with MEL, which has multiple strong binding sites. Therefore, the method reported here allows sensitive determination of MEL in milk products without any interference.

CONCLUSIONS

Melamine is used illegally in protein-rich foods such as dairy products to enhance the protein content, but melamine cannot be detected by Kjeldahl method. Although several methods (e.g., HPLC and GC) have been used to detect melamine in dairy products, these methods are not suitable for routine analysis. For this purpose, we developed a method to use L-cysteine-capped Mn-doped ZnS QD to determine melamine levels in dairy products. The QD were synthesized and identified in the present study; the nanoparticles had excellent phosphorescent properties, and the working mechanism of the QD probe was based on quenching of its phosphorescence intensity. The developed method has some advantages over conventional fluorescence and phosphorescence techniques. First, it avoids interference from autofluorescence or cations such as Na^+ , K^+ , Mg^{2+} , and Ca^{2+} . In addition, it does not require the removal of dissolved oxygen or the addition of surfactant or other chemical modifiers, and no derivatization step is required. The proposed method was used to quantify melamine in cheese, yogurt, cream, coffee creamer, and follow-on milk. Concentrations of melamine could be directly monitored according to the changing of RTP

Table 3. Comparison of the proposed method with different techniques reported for the determination of melamine

Method	Linear range (ng/mL)	Limit of detection (ng/mL)	RSD ¹ (%)	Reference
Proposed method	50–500	5.9	0.1	This work
Fluorescence	25–1,000	19.0	1.6	Huang et al., 2010
HPLC	50–10,000	8.1	0.8	Finete et al., 2014
GC-MS	50–800	10.0	7.9	Pan et al., 2013
Electrochemistry	630–11,350	107.0	1.0	Araujo and Paixao, 2014
Spectroscopy	100–3,780	80.0	3.6	Chansuvarn et al., 2013

¹Relative standard deviation.

intensity of QD. Our proposed method is sensitive, selective, fast, and inexpensive, and it has a low detection limit.

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