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Sensitivity and specificity enhanced enzyme-linked immunosorbent assay by rational hapten modification and heterogeneous antibody/coating antigen combinations for the detection of melamine in milk, milk powder and feed samples

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ABSTRACT

The adulteration of food products with melamine has led to an urgent requirement for sensitive, specific, rapid and reliable quantitative/screening methods. To enhance the sensitivity and specificity of the enzyme-linked immunosorbent assay (ELISA) for the detection of melamine in milk, milk powder and feed samples, rational hapten modification and heterogeneous antibody/coating antigen combinations were adopted. Three melamine derivatives with different length of carboxylic spacer at the end were synthesized and linked to carrier proteins for the production of immunogens and coating antigens. Monoclonal antibody against melamine was produced by hybridoma technology. Under optimal experimental conditions, the standard curves of the ELISAs for melamine were constructed in range of 0.1–100 ng mL^{-1}. The sensitivity was 10–300 times enhanced compared to those in the published literatures. The cross-reactivity values of the ELISAs also demonstrated the assays exhibited high specificity. Five samples were spiked with melamine at different concentrations and detected by the ELISA. The recovery rates of 72.8–123.0% and intra-assay coefficients of variation of 0.8–18.9% ($n=3$) were obtained. The ELISA for milk sample was confirmed by high-performance liquid chromatography with a high correlation coefficient of 0.9902 ($n=6$). The proposed ELISA was proven to be a feasible quantitative/screening method for melamine analysis.

1. Introduction

Melamine (2,4,6-triamino-1,3,5-triazine, $C_3H_6N_6$, Fig. 1) was commonly used as an industrial chemical in the production of melamine–formaldehyde polymer resins for laminates, coatings, commercial filters, glues or adhesives, plastics and flame-retardants [1]. Melamine can be found in food and beverages due to migration from melamine-containing resins [2], or as a metabolite product of cyromazine, an insecticide used on animals and crops [3]. The high nitrogen content of melamine has led to its being used as adulterants in feed and food, presumably to inflate the amount of measurable nitrogen, as determined by Kjeldahl method, producing products that appear to have more protein at a reduced cost. Melamine was originally viewed as nontoxic when administered in the purified form as a supplement [4]; however, it can form lethal kidney stones [5], especially when combined with cyanuric acid [6], due to precipitation of insoluble melamine cyanurate. High and prolonged dietary exposure to melamine results in the formation of bladder stones and increase incidence of urinary bladder tumors. Large outbreaks of nephrotoxic renal failure occurred in dogs and cats attributed to ingestion of melamine-containing pet food in 2004 and 2007 [7,8]. In September 2008, infant formulas that were illegally adulterated with melamine led to health problems for thousands of infants in China [9]. In many countries, the tolerance level for melamine is regulated to be 1 mg kg^{-1} for baby formula and 2.5 mg kg^{-1} for food containing $>15\%$ milk [10]. Therefore, determination of melamine is of biological, clinical, and food industry importance, and sensitive, specific and high throughout analytical methods are needed to determine melamine residues in food and feed, and particularly in dairy products for children.

Many methods including capillary electrophoresis [11], HPLC [12], LC-MS/MS [13,14], GC–MS [15], nuclear magnetic resonance spectroscopy [16], MALDI-MS [17] have been developed for the detection of melamine. These methods are accurate, but they are expensive and time-consuming and often require complicated

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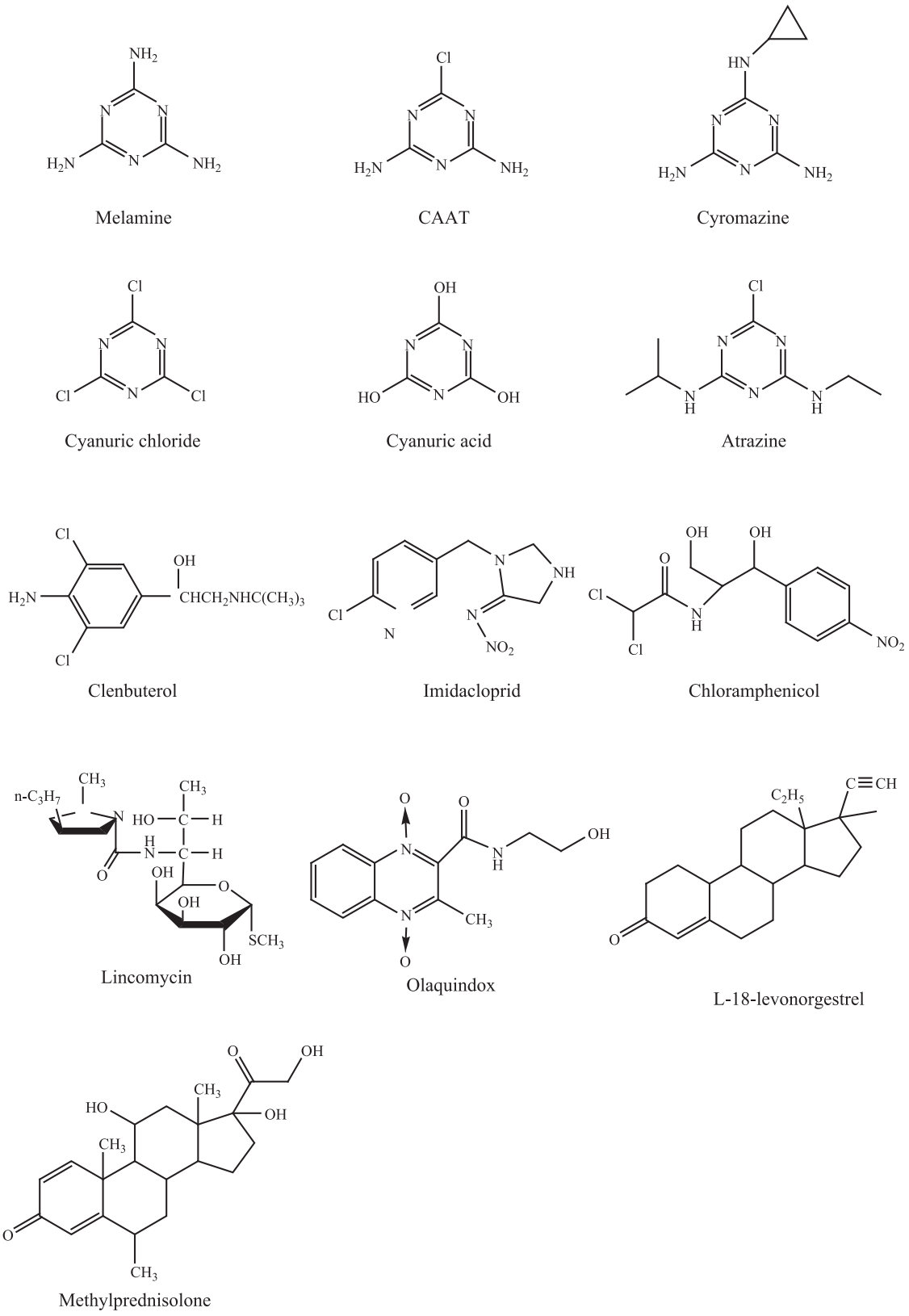

Fig. 1. Molecular structures of melamine and other compounds used for cross-reactivity testing.

sample preparation before analysis. Recently various other techniques such as visual detection by use of gold nanoparticles [18,19] and surface-enhanced Raman spectroscopy [20,21] were proposed for melamine detection. However, these methods suffer from low sensitivity or specificity.

Immunoassays, especially enzyme-linked immunosorbent assays (ELISAs), are analytical methods which are based on the specific interaction between an antibody and corresponding antigen. Immunoassays are generally rapid, high sensitivity and specificity, simple sample preparation, high throughput, and therefore, low cost per

sample. Many ELISAs have been widely used for the determination of low molecular contaminants in the food and environmental analyses [22–26]. In the last years, several ELISAs have been reported for the detection of melamine in raw milk, milk powder, animal feeds and muscle tissues [10,27–30]. Xi et al. and He et al. developed two kinds of melamine ELISAs with the IC_{50} values of 22.6 ng mL^{-1} and 500 ng mL^{-1}, respectively [10,27]. However, in both papers, the cross-reactivity of the ELISA with cyromazine, a triazine pesticide used for fly control in crop production and animal feed to inhibit insect growth, was not indicated. Actually melamine is the main metabolite of cyromazine. Due to the structural similarity to melamine, cyromazine is a major potential matrix factor which is not expected in the immunochemical analysis of melamine. Sun et al. and Wang et al. reports three melamine ELISAs with the IC_{50} values in range of 13.0–70.6 ng mL^{-1}; the cross-reactivity values of the ELISAs with cyromazine were found within 17.1–267.6% [28–30]. Apparently for all published ELISAs mentioned above, both the sensitivity and specificity for the detection of melamine are not so high, e.g. they should be further improved.

The aims of this study are to produce monoclonal antibody against melamine and to develop highly sensitive and specific ELISAs for the detection of melamine in milk, milk powder and animal feeds. In this study, the hapten (e.g. the small molecular analyte, herein is melamine) modification was rationally designed. Three derivatives of melamine with different length of spacer bearing a carboxylic group at the end were synthesized and used to link carrier proteins for the production of immunogens and coating antigens. Monoclonal antibody was produced by hybridoma technology. To improve the sensitivity of the assays, heterogeneous antibody/coating antigen combinations were adopted. The established ELISA was characterized in terms of sensitivity, specificity, accuracy and precision. Under optimal experimental conditions, the proposed ELISA was used for the analysis of fortified food samples and was validated by HPLC method for the analysis of melamine in milk samples.

2. Experimental

2.1. Chemicals

Bovine serum albumin (BSA), ovalbumin (OVA), dimethyl sulphoxide (DMSO), dimethylformamide (DMF), Tween-20, acetonitrile (HPLC grade), casein, 3,3′,5,5′-tetramethylbenzidine (TMB), *N*,*N*-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), Freund's complete and incomplete adjuvants hypoxanthine aminopterin thymidine (HAT), hypoxanthine thymidine (HT) and polyethylene glycol (PEG 4000) were purchased from Sigma (St Louis, MO, USA). Cyanuric acid, cyanuric chloride and succinic anhydride were bought from Sinopharm Chemical Reagent Co. (Shanghai, China). Cyromazine was obtained from Guobang Pharmaceutical Co. (Zhejiang, China). Atrazine was from Zhongshan chemical Co. (Zhejiang, China). 2-chloro-4,6-diamino-1,3,5-triazine (CAAT) was from Acros Organics (Geel, Belgium). Melamine, 4-aminobenzoic acid and 3-mercaptopropionic acid were bought from Chang Zheng Chemical Co. (Chengdu, China). Horseradish peroxidase labeled goat anti-mouse IgG conjugate (HRP-GaMIgG) was obtained from Zhong Shan-Golden Bridge Biological Technology Co. (Beijing, China). RPMI 1640 was bought from GibcoBri (Paisley, Scotland). Cell medium and foetal calf serum were from Minhai (Lanzhou, China). Mouse SP2/0 myeloma cell was bought from the Cell Bank of Chinese Science Academy (Shanghai, China). BALB/C mice were purchased from Experimental Animal Center of Suzhou University (Suzhou, China).

2.2. Apparatus, buffers and solutions

ELISA reader (Sunrise Remote/Touch Screen) and microtiter plate washer (M12/2R) were bought from Columbus plus (Tecan, Grödig, Austria). CO_2 incubator (HF 151 UV) was from HealFore Development Ltd. (Shanghai, China). Spectrophotometer UV-2300 was from Techcomp (Shanghai, China). Microtiter plate shaker (KJ-201C Oscillator) was from Kangjian Medical Apparatus, Co., Ltd. (Jiangsu, China). 96-well polystyrene microtiter plates were from Haimen Plastic (Jiangsu, China). Deionized-RO water supply system (Dura 12FV) was purchased from THE LAB Com. (USA).

Buffers and solutions: (1) coating buffer: 0.05 mol L^{-1} carbonate buffer, pH 9.6; (2) coating antigen stock solution: 1 mg mL^{-1} of coating antigen prepared with coating buffer; (3) assay buffer: 0.01 mol L^{-1} phosphate-buffered saline (PBS) pH 7.4, containing 145 mmol L^{-1} NaCl; (4) washing buffer (PBST): assay buffer with 0.1% (v/v) of Tween-20; (5) blocking solution: 1% of casein in assay buffer; (6) acetate buffer: 100 mmol L^{-1} sodium acetate acid buffer, pH 5.8; (7) substrate solution (TMB+H_2O_2): 200 μL of 10 mg mL^{-1} TMB dissolved in DMSO, 3.5 μL of 30% H_2O_2 and 1 mL of acetate buffer were added to 20 mL of pure water; (8) stop solution: sulfuric acid (5%). (9) melamine standard solutions at the concentrations of 0, 0.1, 0.3, 1.0, 2.0, 5.0, 10 and 100 ng mL^{-1} were prepared by diluting the stock solution (1 mg mL^{-1}, by dissolving melamine in DMSO) with water.

2.3. Synthesis of melamine derivatives

In this study, to prepare immunogens and coating antigens, three melamine derivatives, e.g. 4-[(4,6-diamino-1,3,5-triazin-2-yl) amino]benzoic acid (I), 3-[(4,6-diamino-l,3,5-triazin-2-yl)thio] propanoic acid (II) and 4-[(4,6-diamino-1,3,5-triazin-2-yl)amino] butyric acid (III), with different length of spacer bearing a carboxylic group at the end were synthesized. The synthetic processes were illustrated in Fig. 2.

The synthesis of derivative I [(Fig. 2(a)] was performed as described in the literature with a small modification [31]. Briefly, 5 mmol of CAAT was suspended in 100 mL of absolute methanol, then 5.4 mmol of 4-aminobenzoic acid and 10.8 mmol of KOH dissolved in 10 mL of absolute methanol were added dropwise. The reaction was refluxed for 12 h and the process of the reaction was monitored by silica gel thin layer chromatography (TLC). The reaction mixture was filtered, and the resultant was washed with absolute ethanol followed by distilled water (4 °C), then the white crude product was obtained. The final product was purified using silica gel chromatography (methanol:dichloromethane, 1:19).

The synthesis of derivative II was similar to that for derivative I [(Fig. 2(b)]. The starting reagent was also CAAT, but the derivatizing reagent was 3-mercaptopropanoic acid and reaction solvent was ethanol.

In the process of synthesizing derivative III [(Fig. 2(c)], the starting reagent was melamine instead of CAAT, and the derivatizing reagent was succinic anhydride. Briefly, 0.5 mmol of melamine was reacted with 0.5 mmol of succinic anhydride in 5 mL pyridine overnight at room temperature. The extent of the reaction was ascertained using TLC. After the reaction was complete, the pyridine was evaporated under a stream of nitrogen, then the white crude product was obtained. The final product was purified using silica gel chromatography (methanol:dichloromethane, 1:19).

2.4. Preparation of immunogens and coating antigens

Via the carboxylic acid group, the melamine derivatives were conjugated to carrier proteins by the DCC/NHS activation method as described in the literature [23,24]. Briefly, equimolar amounts (0.15 mmol) of melamine derivative I (or II, or III), NHS, and DCC were dissolved in 300 μL of DMF together with 20 μL of DMSO and the mixture was incubated overnight at 25 °C. The solution was centrifuged at 13,400 rpm for 10 min and the supernatant was added slowly to 100 mg of protein (BSA or OVA) dissolved in 5 mL

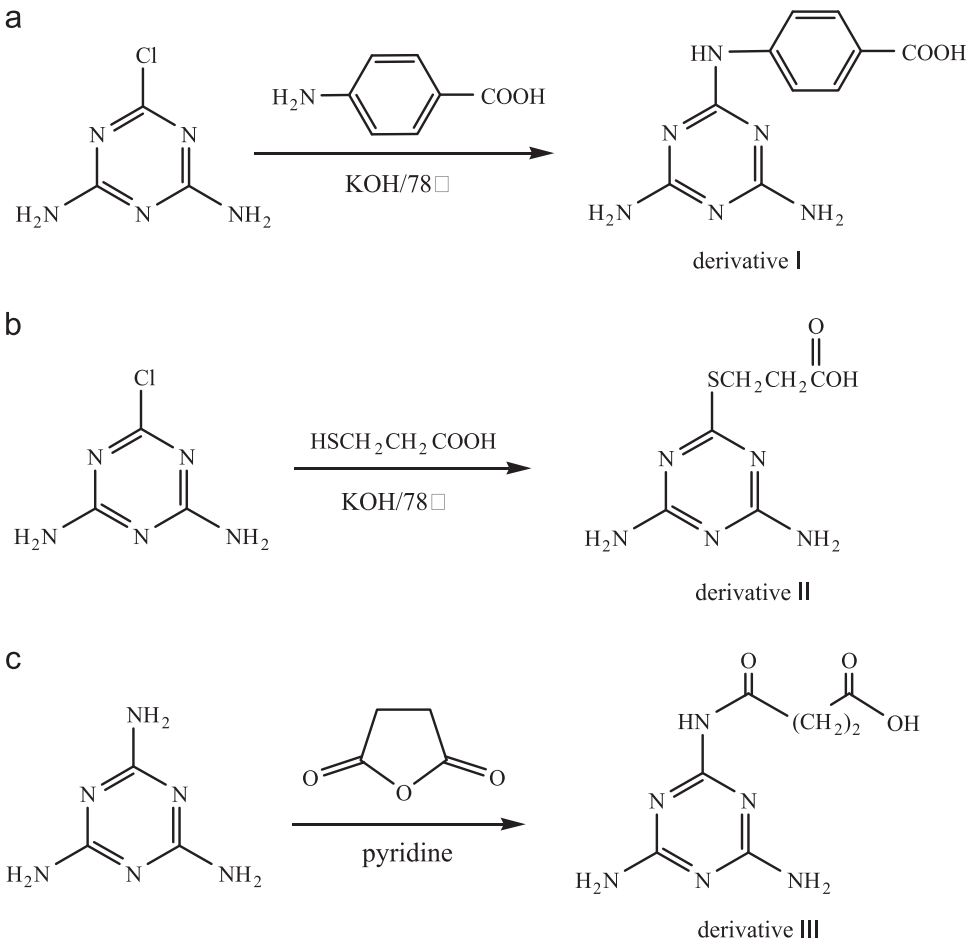

Fig. 2. Synthesis of melamine derivative I (a), derivative II (b) and derivative III (c).

of 0.13 mol L^{-1} $NaHCO_3$ under stirring. After incubation for 24 h at 25 °C, the solution was centrifuged and the supernatant was intensively dialyzed in 0.01 mol L^{-1} $(NH_4)_2CO_3$ for 4 days with several changes of the dialyzing buffer solution. Finally, the derivative-protein conjugates were lyophilized and stored in the refrigerator until use. The formed derivative I-BSA, derivative II-BSA and derivative III-BSA were used as immunogens for antibody preparation, while derivative I-OVA, derivative II-OVA and derivative III-OVA were served as coating antigens for ELISA establishment.

2.5. Production of monoclonal antibody

BALB/c mice were immunised with 100 μg of immunogen in Freund's complete adjuvants. In next two sequential booster immunizations, 100 μg of immunogen emulsified with the same volume of Freund's incomplete adjuvant was given to each mouse in the same way at 2-week intervals after the initial immunization. The mice were given the final booster injection intraperitoneally without adjuvant before cell fusion. Tail bleeding was carried out to determine specific anti-melamine antibody serum titre before sacrificing the animals for spleens. Generations of hybridomas were carried out as described elsewhere [25]. In brief, isolated splenocytes were fused with the mouse SP2/0 myeloma cells at the ratio of 10:1 in the presence of 50% polyethylene glycol 4000 and selection for hybridomas were carried out in HAT media. Culture supernatants from each clone were subjected to screening by indirect competitive ELISA. The hybridomas which produced antibody-recognizing with melamine were subcloned for three times using the limiting dilution method. Stable antibody-producing clones were expended until monoclones were obtained. Monoclonal antibodies were then produced in mouse ascites and purified with saturated ammonium precipitation.

2.6. Indirect competitive ELISA

An indirect competitive ELISA format was adopted for analyzing melamine. The ELISA procedures were as follows. Coating antigen stock solution (1 mg/mL) was diluted with carbonate buffer, pH 9.6, and 200 μL/well added to a 96-well microtiter plate. The plate was incubated overnight at 4 °C and then washed with PBST (350 μL/well) three times using an automated plate washer. Some binding sites not occupied by the coating antigen were then blocked by the blocking buffer (280 μL/well) for 1 h at room temperature. After the plate was washed as before, standard solutions or samples in triplicate (100 μL/well) and diluted antibody (100 μL/well) were added and incubated for 1 h at room temperature. After washing, HRP-GaMIgG was added (200 μL/well) and the plate incubated for 1 h at room temperature. Then, the plate was washed and the substrate solution (200 μL/well) added. After incubation with shaking for about 15 min, sulfuric acid (5%, 80 μL/well) was added and the absorbance measured at 450 nm using a microplate reader. Calibration curves were constructed in the form of $(B/B_0) \times 100\%$ vs. log C, where B and B_0 was the absorbance of the analyte at the standard point and at zero concentration of the analyte, respectively.

2.7. Cross-reactivity

The specificity of the produced monoclonal antibody was investigated by cross-reactivity (CR) experiments. Twelve compounds

including five structurally related compounds (CAAT, cyromazine, cyanuric acid, cyanuric chloride, atrazine) and seven other compounds (clenbuterol, imidacloprid, chloramphenicol, lincomycin, olaquindox, levonorgestrel, methylprednisolone) were selected for testing CR. The molecular structures of the CR testing compounds were illustrated in Fig. 1. Standard solutions of testing compounds (0.001–10,000 ng mL^{-1}) were prepared and subjected to ELISA procedures. CR was expressed as IC_{50} value based on 100% response of melamine, i.e. CR (%)=(IC_{50} of melamine)/(IC_{50} of testing compound) × 100%. The IC_{50} value can be considered as a measure (inverse) of the affinity of an antibody for a given analyte.

2.8. Fortification experiment

Five samples including pure milk I, pure milk II, milk powder, chicken feed and pig feed were collected from local supermarket in Suzhou (China). To obtain the accurate and precise values of the ELISA, all collected samples were spiked with melamine at different concentrations and detected by ELISA. Pure milk and milk powder can be directly applied for fortification, while chicken feed and pig feed need to be homogenized before fortification. The fortification experiment was performed as follows. For pure milk, 1 mL of milk was individually put into glass tubes with glass stoppers. Appropriate amount of melamine stock solution (1 mg mL^{-1}) was added into the tubes to prepare a final concentration of 1.0, 2.5 and 3.0 μg mL^{-1}. Vigorously shaking the samples for 5 min and staying at room temperature for 1–2 h, then 0.1 mL of the fortified sample was taken and diluted with 0.01 mol L^{-1} PBS at 1:100 to 1:500. The diluted milks were directly applied to ELISA. For milk powder, 1 g of sample was used and appropriate amount of melamine stock solution was added. The final concentration fortified with melamine was at 1.0, 2.5 and 3.0 μg g^{-1}. Ten milliliter of 0.01 mol L^{-1} PBS was added and shaken vigorously for 5 min. After staying at room temperature for 1–2 h, one milliliter of fortified sample was taken and diluted with 0.01 mol L^{-1} PBS at 1:30 to 1:50. The concentration of melamine in diluted samples was measured by the proposed ELISA. For chicken feed and pig feed, 1 g of feed sample was taken and the final concentration fortified with melamine was at 15, 20 and 30 μg g^{-1}. Ten milliliter of trichloroacetic acid (TCA, 1%) was added. The mixture was sonicated for 20 min and centrifuged at 10,000*g* for 10 min at 4 °C. Then, 1 mL of supernatant was taken and diluted with 0.01 mol L^{-1} PBS at 1:200 to 1:400 before analyzing by ELISA. For each sample, three separate extractions were performed and each sample was determined in triplicate. Unspiked samples were extracted in the same way and used as blanks.

2.9. HPLC-UV analysis

HPLC was performed according to the procedure of reference [32]. Melamine standard solutions or sample extracts were passed through a 0.45 μm cellulose acetate membrane filter prior to HPLC detection. A HPLC system (Alltech, USA) with a C_{18} column (250 × 4.6 mm, 5.0 μm particle size, Alltech, USA) was equilibrated with the mixture of mobile phase A (acetonitrile): mobile phase B (10:90, v/v) at a flow rate of 1 mL min^{-1}, where the mobile phase B was prepared by dissolving 2.16 g of octane sulfonic acid sodium and 2.10 g of citric acid in 1000 mL of H_2O and the pH value was adjusted to be 3.0. The volume of standard or extract in each analysis was 20 μL. Melamine was monitored at 240 nm by UV detector. The calibration curve for melamine was constructed with standards of 0.4, 0.8, 2, 4, 8, 20, 60 μg mL^{-1}.

In this study, to validate the applicability of the established ELISA for the detection of melamine, one kind of sample (pure milk I) was selected and measured by HPLC and ELISA simultaneously. 2 mL of milk were taken and fortified with melamine at the final concentration of 0.5, 2.0, 4.0, 8.0, 20 and 40 μg mL^{-1}. Fifteen milliliters of TCA (1%) together with 5 mL of acetonitrile were added. The mixture was sonicated for 10 min, vigorously shaken for 10 min, followed by centrifugation at 10,000*g* for 10 min at 4 °C. The supernatant was filtered through the filter paper which was previously wetted by 1% TCA. All filtrate was collected and supplemented with 1% TCA to final volume of 25 mL. Two milliliters of filtrate were taken and mixed with equivalent pure water for clean-up procedure which was carried out on MCX solid phase extraction column (Waters, Japan). The MCX SPE column was preconditioned by passing 3 mL of methanol, followed by 3 mL of pure water through the column. Then, 4 mL of the TCA/acetonitrile extract was slowly passed through the MCX column at a flow rate of 1 mL min^{-1}. After washing with 3 mL of pure water and 3 mL of methanol, the analytes were eluted with 3 mL of ammonia–methanol solution (5%, v/v). The solution was evaporated to dryness in a 50 °C water bath under nitrogen. The residue was reconstituted with 400 μL of the mixture of mobile phase A and mobile phase B, and simultaneously measured by HPLC and ELISA.

3. Results and discussion

3.1. Synthesis of melamine derivatives

To prepare highly specific antibody against small molecular analyte, generally the molecular structure of the hapten should be left unchanged as far as possible. Melamine has a ringed symmetrical structure with three equal-active amidogens which might be used as coupling positions. It will be desired that only one amidogen be used for derivation. This will be achieved by selecting appropriate starting reagent such as CAAT or by controlling the molar ratio between the starting reagent and the derivative reagent. In CAAT, the active chloride group was capable of reacting relatively easy with the primary amino of a spacer arm reagent such as 4-aminobenzoic acid or the sulphydryl group of a spacer arm reagent such as 3-mercaptopropionic acid. On the other hand, by keeping the molar ration between melamine and derivative reagent (such as glutaraldehyde, succinic anhydride, halogenated carboxylic acids or their esters) at 1:1, one spacer arm derivative of melamine might be prepared. Using synthetic approaches described above, in this study, three melamine derivatives with different length of spacer bearing a carboxylic group at the end were synthesized. More different derivatives will greatly increase the probability to produce highly specific antibodies against the target analyte and to establish a high sensitive ELISA through the optimization of different combination of antibody and coating antigen [24].

The structures of the melamine derivatives were confirmed by NMR method. The data from NMR method are follows: derivative I: ^{1}H NMR (DMSO-d6, 400 MHz): 2.09 (*s*, 2H), 6.09 (*s*, 4H), 7.77–7.91 (*t*, 4H), 9.2 (*s*,1H); derivative II: ^{1}H NMR (DMSO-d6, 400MHz): 2.64 (*t*, 2H), 3.14 (*t*, 2H), 6.9 (br, 4H); derivative III:^{1}H NMR (DMSO-d6, 400 MHz): 2.21, 3.20 (*t*, 2H), 6.49 (*s*, 4H); 9.67 (*s*, 1H). The NMR spectroscopic data demonstrated that the three kinds of melamine derivatives were successfully synthesized.

3.2. Production of monoclonal antibody

Six BALB/C mice were immunised with derivative I-BSA, derivative II-BSA and derivative III-BSA. After third injection, it was observed that antisera collected from six immunised mice displayed high affinity binding with coating antigen. Three days after the final

booster injection, the mice were killed. The spleen cells were removed and used for the fusion experiments. After incubated for 2 weeks, the supernatants from hybridoma cells were screened by an indirect competitive ELISA. The hybridomas which produced antibody recognition with melamine were subcloned for three times using the limiting dilution method. It was found that seven hybridoma cells producing antibody recognition with melamine. However when melamine was as a competitor, we found that only the supernatant from the hybridoma clone using melamine derivative I-BSA as an immunogen displayed a strong inhibition tested by an indirect competitive ELISA, while the supernatant from other hybridoma clones using derivative II-BSA and derivative III-BSA as immunogens showed a week inhibition. Therefore the mAb from the hybridoma clone based on derivative I-BSA as immunogen was used for the establishment of ELISA. Among three immunogens, the possible reason that only derivative I-BSA produce highly specific antibody against analyte might be partly due to the higher rigidity of the arm spacer (benzene ring) in derivative I-BSA which can improve the immunogenicity [33].

3.3. Optimization of ELISA conditions

To enhance the sensitivity, the assay conditions including type and concentration of the coating antigen, dilution of the antibody and secondary labeled antibody, etc. should be carefully optimized. In the present investigation, it was performed according to two criteria, i.e. (1) to get an IC_{50} value as low as possible and (2) an absorbance in the range of 0.8–1.5 absorption units for the zero standard concentration. In this study, based on three different coating antigens (derivative I-OVA, derivative II-OVA and derivative III-OVA), three possible combinations were tested (Table 1). The antibody/derivative I-OVA was considered to be homogeneous combination as the antibody was produced based on derivative I-BSA as immunogen; while other two belonged to heterogeneous combinations. The experimental conditions including the concentration of coating antigen and the dilution of antibody and HRP-GaMIgG were optimized for each combination. It was seen from Table 1 that the coating antigen concentration, antibody dilution and HRP-GaMIgG dilution varied in the range of 100–2000 ng mL^{-1}, 1:20,000–1:50,000 and 1:5000–1:10,000, respectively.

3.4. Sensitivity of the ELISAs

Under optimal assay conditions, the ELISA standard curves of three antibody/coating antigen combinations for melamine detection were constructed in concentrations of 0.1–100 ng mL^{-1} (Fig. 3). The sensitivity was expressed by IC_{50} value, the lower of the IC_{50} value, the higher sensitivity of the assay would be. It was seen from Fig. 3 that the IC_{50} value of ELISA based on homogeneous combination (e.g. antibody/derivative I-OVA) was 19.1 ng mL^{-1}, while the IC_{50} values of ELISAs based on heterogeneous combinations (e.g. antibody/derivative II-OVA and antibody/derivative III-OVA) were found to be 1.70 and 3.90 ng mL^{-1}, respectively. Apparently the sensitivity of the ELISA based on heterogeneous format is 5–10 times higher than that based on homogeneous format. This might be due to the fact that as the antibody was produced based on derivative I-BSA as immunogen, the recognition affinity of the antibody toward derivative II-OVA or derivative III-OVA will be lower than that toward the derivative I-OVA. In other words, in heterogeneous format, the antibody will have a higher affinity toward the analyte in comparison to the coating antigen, leading to a higher sensitivity. Obviously, the types of antibody/coating antigen combination play an important role in assay sensitivity. In this study, it is a great advantage for us to have synthesized three different melamine derivatives instead of only one (or two) so that we could prepare different coating antigens and sufficiently optimize antibody/coating antigen combinations to improve assay sensitivity.

It was also noticed that based on heterogeneous formats, the IC_{50} values achieved in this study were about 10–300 times lower than those in published papers [10,27–30], indicating high sensitivity of our ELISAs. On the other hand, due to the sensitivities of ELISAs of the two heterogeneous formats are very close, these two ELISAs using derivative II-OVA and derivative III-OVA as coating antigens were employed in further experiment.

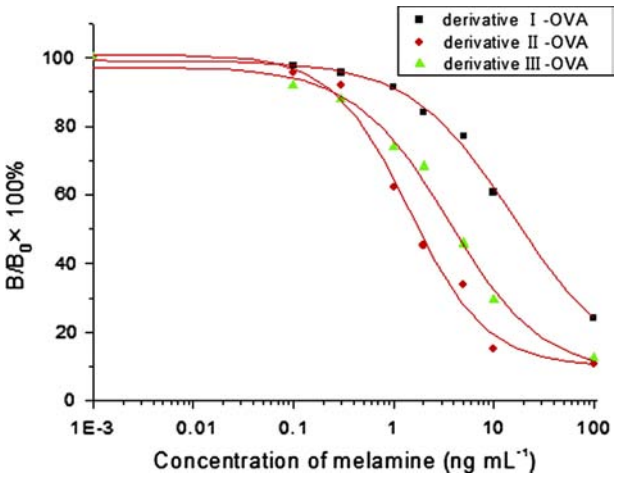

Fig. 3. Standard curves of ELISAs for melamine based on different coating antigens. (■) derivative I-OVA, IC_{50} = 19.1 ng mL^{-1}; (●) derivative II-OVA, IC_{50} = 1.70 ng mL^{-1}; (▲) derivative III-OVA, IC_{50} = 3.90 ng mL^{-1}.

Table 1
Optimized ELISA conditions including coating antigen, antibody and HRP-GaMIgG.

Coating antigen			Antibody	HRP-GaMIgG
Name	Dilution	ng/well	Dilution	Dilution
Derivative I-OVA	1:10000	100	1:50,000	1:5,000
Derivative II-OVA	1:1000	1000	1:50,000	1:5,000
Derivative III-OVA	1:500	2000	1:20,000	1:10,000

3.5. Specificity of the assay

The assay specificity was evaluated by CR of the ELISAs with five structurally related compounds (e.g. CAAT, cyromazine, cyanuric acid, cyanuric chloride and atrazine) and seven other compounds (clenbuterol, imidacloprid, chloramphenicol, lincomycin, olaquindox, levonorgestrel and methylprednisolone). The IC_{50} and CR values of the ELISAs for each compound were given in Table 2. It was seen from Table 2 that, in the case of derivative II-OVA as a coating antigen, the CR values of the ELISA with CAAT and cyromazine were 6.3 and 10.5%, respectively; there was no cross-reactivity (CR < 0.01%) of the ELISA with other testing compounds, including three structurally related compounds (cyanuric acid, cyanuric chloride and atrazine). Some extent cross-reactivity (CR < 11%) of the ELISA with CAAT and cyromazine might be mainly due to the similarity of the molecular structure of CAAT and cyromazine to that of analyte. No cross-reactivity (CR < 0.01%) of the ELISA with cyanuric acid, cyanuric chloride and atrazine would be expressed by the fact that, although cyanuric acid, cyanuric chloride and atrazine contain the same 1,3,5-triazine frame as that of melamine, however, the replacement of all three amino groups in original melamine with chloro-, hydroxy- or alkylamino-groups in these three molecules make them to be hard recognized by the produced mAb using derivative I-BSA as an

Table 2
Cross-reactivities (CR) of the ELISAs (using derivative II-OVA and derivative III-OVA as coating antigens) with melamine and other compounds.

Compound	Derivative II-OVA		Derivative III-OVA	
	IC_{50} (ng mL^{-1})	CR (%)	IC_{50} (ng mL^{-1})	CR (%)
Melamine	1.70	100	3.90	100
CAAT	27.0	6.30	76.7	5.10
Cyromazine	16.1	10.5	47.9	8.20
Cyanuric acid	> 10,000	< 0.01	> 10,000	< 0.01
Cyanuric chloride	> 10,000	< 0.01	> 10,000	< 0.01
Atrazine	> 10,000	< 0.01	> 10,000	< 0.01
Clenbuterol	> 10,000	< 0.01	> 10,000	< 0.01
Lmidacloprid	> 10,000	< 0.01	> 10,000	< 0.01
Chloramphenicol	> 10,000	< 0.01	> 10,000	< 0.01
Lincomycin	> 10,000	< 0.01	> 10,000	< 0.01
Olaquindox	> 10,000	< 0.01	> 10,000	< 0.01
Levonorgestrel	> 10,000	< 0.01	> 10,000	< 0.01
Methylprednisolone	> 10,000	< 0.01	> 10,000	< 0.01

Table 3
The results of recovery and coefficient of variation for melamine determination from five fortified samples measured by ELISA.

Sample	Conc. fortified (μg mL^{-1} or μg g^{-1})	Conc. measured ± SD (μg mL^{-1} or μg g^{-1})	Recovery (%)[a]	Intra-assay CV (%, $n=3$)	Inter-assay CV (%, $n=3$)
Pure milk I	0	0.09			
	1.0	1.08 ± 0.14	99.0	13.0	8.0
	2.5	2.20 ± 0.24	84.4	10.9	4.9
	3.0	3.36 ± 0.47	109.0	14.0	20.8
Pure milk II	0	0.06			
	1.0	0.86 ± 0.10	80.0	11.6	5.7
	2.5	1.88 ± 0.20	72.8	10.6	11.8
	3.0	2.90 ± 0.36	94.7	12.4	19.7
Milk powder	0	0.08			
	1.0	1.31 ± 0.11	123.0	8.4	19.1
	2.5	2.42 ± 0.46	93.6	18.9	16.4
	3.0	3.48 ± 0.07	113.3	2.0	2.6
Chicken feed	0	1.3			
	15	16.2 ± 1.1	99.3	7.0	9.1
	20	21.3 ± 3.1	100.0	14.4	17.8
	30	31.6 ± 3.0	101.0	9.5	3.9
Pig feed	0	0.6			
	15	16.6 ± 0.1	106.7	0.8	3.9
	20	18.4 ± 2.4	89.0	13.2	10.1
	30	30.2 ± 3.2	98.7	10.7	10.3

[a] Recovery (%)=[(Conc. measured-blank)/Conc. fortified] × 100%.

immunogen. In the case of derivative III-OVA as a coating antigen, similar CR results were observed in which the CR of the ELISA with CAAT and cyromazine were 5.1 and 8.2%, respectively; there was also no CR of the ELISA with other testing compounds.

It is apparent that the cross-reactivity values of the above two ELISAs are much lower than those in published papers [10,27–30], indicating high specificity of our ELISAs. On the other hand, because the specificity of the ELISA using derivative III-OVA as a coating antigen was slightly higher than that of ELISA using derivative II-OVA as a coating antigen, thus the ELISA using derivative III-OVA as a coating antigen was selected for further experiment.

3.6. Accuracy and precision of the assay

Five collected samples without fortification were analyzed by ELISA. The melamine contents in pure milk I, pure milk II, milk powder, chicken feed and pig feed measured by the proposed ELISA were found to be 0.09 μg mL^{-1}, 0.06 μg mL^{-1}, 0.08 μg g^{-1}, 1.3 μg g^{-1} and 0.6 μg g^{-1}, respectively. Small amount of melamine in these unfortified samples detectable by the proposed ELISA was considered the blanks of the matrices.

To test accuracy and precision of the ELISA, five collected samples were fortified with melamine at different concentration level and analyzed by ELISA. The results of accuracy and precision of the assay for the detection of melamine in fortified samples in triplicate were given in Table 3. The recovery rates of 72.8–123.0% and intra-assay coefficients of variation of 0.8–18.9% ($n=3$) were obtained. The inter-assay coefficients of variation were 2.6–20.8% ($n=3$). These results demonstrated the applicability of the ELISA for detecting melamine in different matrices.

3.7. Validation of the assay with HPLC

The HPLC calibration curve for melamine was constructed in the range of 0.4, 0.8, 2, 4, 8, 20, 60 μg mL^{-1}. The retention time of melamine was 15 min. The linear equation of the HPLC standard curve for melamine detection was $Y=27{,}879x+13{,}493$ ($R=0.9997$, $n=7$).

To validate the applicability of the ELISA, one kind of sample (pure milk I) was fortified with melamine at the final concentration of 0.5, 2.0, 4.0, 8.0, 20 and 40 μg mL^{-1} . After sample pretreatment, the extracts were measured by HPLC and ELISA simultaneously. The comparison of the ELISA with the HPLC was shown in Fig. 4. A good correlation between HPLC (x) and ELISA (Y) was obtained with the linear regression equation of $y=1.5589x-1.4004$ ($R=0.9902$, $n=6$). These results suggested that melamine in food and feed samples could be simply, rapidly and accurately detected by the proposed ELISA.

4. Conclusions

In summary, in this study, using CAAT and melamine as starting reagents, three melamine derivatives with different length of

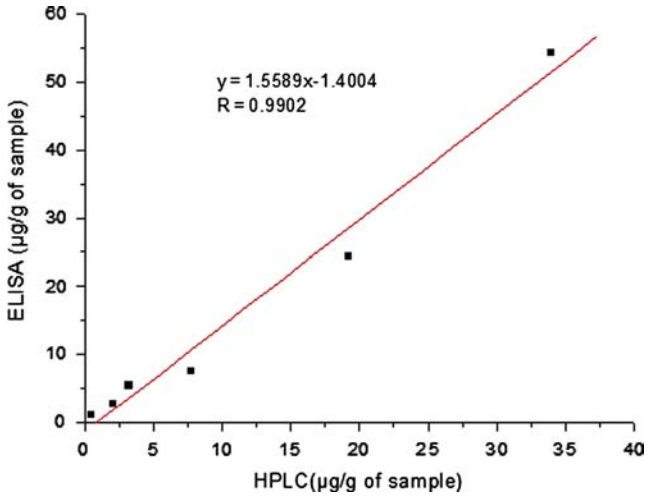

Fig. 4. Correlation between the proposed ELISA and HPLC for the detection of melamine in spiked samples.

carboxylic spacer were synthesized. A mAb against melamine was successfully produced. Highly sensitive and specific ELISAs using heterogenous antibody/coating antigen combinations were established. At optimal assay conditions, the IC_{50} values of the ELISAs for melamine were 1.70–3.90 ng mL^{-1}. The cross-reactivities of the assays with CAAT and cyromazine were within 5.1–10.5% and no cross-reactivity with other structurally related and unrelated compounds was found. Both the sensitivity and specificity of the assays were higher than those in published papers. Five samples were fortified with melamine and detected by the assay. The results of recovery and coefficient variation were accepted. The assay was also validated by HPLC with good correlation. The proposed ELISA could be a feasible quantitative/screening method for melamine analysis in milk, milk powder and animal feed samples.

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