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ARTICLE

Preparation of Monoclonal Antibody for Melamine and Development of an Indirect Competitive ELISA for Melamine Detection in Raw Milk, Milk Powder, and Animal Feeds

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Melamine (MEL) has been involved in several food recalls after the discovery of severe kidney damages in children and pets poisoned by melamine-adulterated food. To detect MEL residue in foods and animal feeds, an indirect competitive ELISA (cELISA) method was developed in this study based on preparation of monoclonal antibodies (MAbs) to MEL. The immunogen was prepared by linking MEL hapten with carrier protein via carbodiimide method. The method is applicable in the range of 5.0−135.0 μg L^{-1} MEL in buffer solution, with an IC_{50} value of 22.6 ± 1.9 μg L^{-1}. The MAbs showed high specificity with low cross-reactivity (≤1%) toward cyanurate, ammelide, and ammeline. The method was utilized in the detection of MEL in raw milk, milk powder, and animal feeds, with detection limits of 0.1 mg L^{-1} for milk, 0.2 mg kg^{-1} for milk powder, and 0.5 mg L^{-1} for feeds. The recovery ratio was 79−110% for all matrices. The intra-assay and interassay coefficients of variation were <12.0 and <13.0%, respectively. Finally, the application of the cELISA in quantity evaluation of MEL in various feeds from local markets was evaluated and discussed.

KEYWORDS: Melamine; drug residue; monoclonal antibody; ELISA

INTRODUCTION

Melamine (1,3,5-triazine-2,4,6-triamine, MEL) (**Figure 1**), a triazine-based chemical used in the manufacture of plastic resins, was identified as an adulterant. Melamine and cyanuric acid (CYA) are both used as adulterants in feed and food because they increase the nitrogen content, as determined by Kjeldah method, producing products that appear to have more protein at a reduced cost (*1*). In March 2007, numerous pet foods in the United States were recalled after dogs and cats consuming the products suffered from renal failure (*2*). Recent research showed that MEL combined with CYA could cause the formation of insoluble crystals in the kidneys of animals and infants (*3*). Therefore, melamine adulteration was also a problem for human consumption. In September 2008, several companies in China were implicated in a scandal involving milk and infant formula adulterated with MEL, leading to kidney stones and other renal failure, especially for infants. Through December 2008, nearly 50000 infants had become ill and 6 infants died. As a concequence, MEL is strictly prohibited by the U.S. Food and Drug Administration (FDA) as a source of nonprotein nitrogen in food or animal feed. CYA is also not approved by the U.S. FDA in hog, chicken, fish, or aquaculture feeds (*4*). In many countries, the tolerance level for MEL is regulated to be 1 mg kg^{-1} for baby formula and 2.5 mg kg^{-1} for food containing >15% milk. Thus, a sensitive method to detect MEL in animal feed and food, especially milk, was required to control the products intended for human consumption.

Many analytical techniques have been developed to detect MEL, such as Raman spectroscopy (*5*), liquid chromatography (LC) (*6*, *7*), gas chromatography (GC) (*8*), HPLC-MS/MS (*9*), zwitterionic hydrophilic interaction chromatography followed by tandem mass spectrometry (*10*, *11*), and capillary zone electrophoresis (CE) (*12*). Instrumental methods such as HPLC, GC, and CE are accurate, but they are expensive and time-consuming and often require complicated sample preparation before analysis. On the contrary, immunoassays are generally rapid, sensitive, selective, and cost-effective (*13*). Several commercial ELISA test kits to detect MEL are available, such as Abraxis Melamine Plate kit, Beacon Melamine plate Kit, CUSABIO Melamine ELISA Kit, and Romer Laboratories AgraQuant Melamine Sensitive Assay. All of these kits are based on polycolonal antibodies toward MEL, among which the Abraxis Melamine Plate kit displays the most sensitive results with an IC_{50} value and limit of detection (LOD) of 100 μg L^{-1} and 9 μg L^{-1} of MEL in phosphate buffer solution, respectively, and a LOD value of 1 mg L^{-1} MEL in dog food (*13*).

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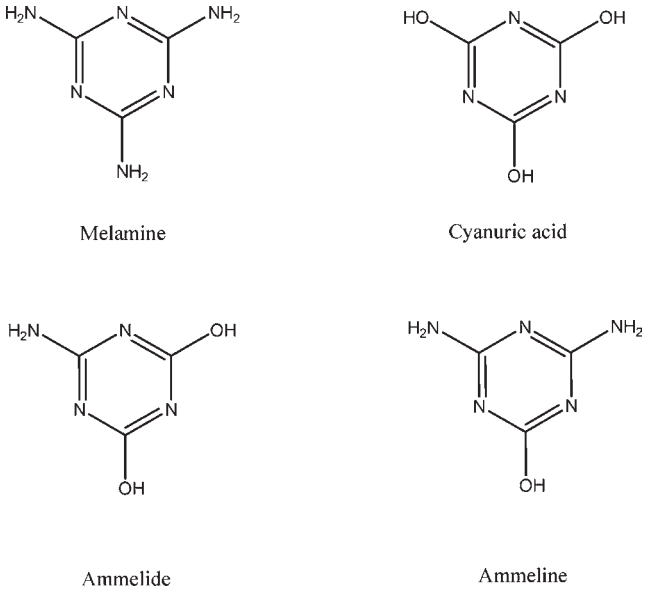


Figure 1. Structures of melamine and structurally related compounds.

The purpose of this investigation is to prepare monoclonal antibodies (MAbs) to MEL and develop an indirect competitive enzyme-linked immunosorbent assay (cELISA) for efficiently monitoring and quantifing MEL in raw milk, milk powder, and animal feeds. This is the first paper to describe the preparation of a MAb toward MEL and its use in detecting MEL in foods. The cELISA method developed in this paper is a hopeful alternative to chromatography spectrometry for regulatory analysis of melamine in milk and animal feeds and helpful to improve the sensitivity and specificity of commercially available ELISA melamine kits.

MATERIALS AND METHODS

Chemicals and Instruments. Melamine was purchased from a reagent company in Jinan (Shandong, China). Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete and incomplete adjuvants (cFA and iFA, respectively), 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*,*N*-dimethylformamide (DMF), *tert*-butyl bromoacetate, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO). *N*-Hydroxysuccinimide (NHS) was obtained from Cxbio Biotechnology Ltd. (Shanghai, China). Goat anti-mouse IgG−horseradish peroxidase (HRP) conjugate was prepared in our laboratory. Carbamide peroxide, tetramethylbenzidine (TMB), and other reagents were of chemical grade supplied by Guangmang Chemical Co. (Jinan, China). The cELISA was carried out with high-binding polystyrene 96-well microtiter plates from Greiner Bio-One GmbH (Frickenahusen, Germany) and measured with a GF-3000 microplate reader from Ruicong Shanghai Technology Development Co., Ltd. (Shanghai, China). A refrigerated centrifuge for buffer preparation (Biofuge stratos, Heraeus) was used. LC-MS analysis was carried out by Agilent 1100 series LC-MSD high-performance ion trap mass spectrometers (LC-MSD-TRAP-VL, Agilent Technologies, Inc., Palo Alto, CA).

Buffers and Solutions. The following solutions were used in this study: coating buffer, 0.1 mol L^{-1}; carbonate buffer (pH 9.3), 30 mmol L^{-1} Na_2CO_3 and 70 mmol L^{-1} $NaHCO_3$; blocking solution, 10% BSA in 0.1 mol L^{-1} sodium barbitone−HCl buffer (pH 8.6); washing buffer, 0.2 mol L^{-1} PBS (containing 0.2 mol L^{-1} NaH_2PO_4 and 0.2 mol L^{-1} Na_2HPO_4, pH 8.2) with the addition of 0.03% sodium azide and 2.0% Tween-20 (w/w); color development substrate solutions A and B, carbamide peroxide and tetramethylbenzidine (TMB), respectively; stopping solution, 2 mol L^{-1} HCl; reconstituted solution used for matrix treatment, 0.2 mol L^{-1} PBS solution (pH 6.6) containing 8% (w/w) DMSO.

Preparation of MEL Hapten. Half a gram of MEL was placed into a 50 mL round-bottom flask, and then 35 mL of DMF was added to completely dissove MEL. To this solution were added 0.44 g of KOH and 0.12 g of anhydrous NaI, followed by the addition of 0.65 mL of *tert*-butyl bromoacetate dropwise. Then the reaction was performed at 65 °C for 10 h. After that, the reaction mixture was cooled to ambient temperature, diluted with 35 mL of H_2O, and extracted by 70 mL of ethyl acetate. The organic phase was dried by rotary evaporator under vacuum. Then the yellow oily product was purified via silica gel column chromatography, with ethyl acetate/petroleum ether (2:1, v/v) as eluted solution to get intermediate product (**Figure 2**, Scheme I). After that, 0.32 g of the product was dissolved in 5 mL of methanol, followed by the addition of 5 mL of TFA. The solution was stirred at room temperature for 20 h. Then, the reaction mixture was dried to get an oily product. The mixture solvent of methanol and ethyl acetate (1:1, v/v) was added to dissolve the oily product. To this solution, petroleum ether was gradually added until white turbidity appeared. The mixture was heated to be clear and cooled to get a white precipitate. The white precipitate was collected to get MEL hapten (**Figure 2**, Scheme II).

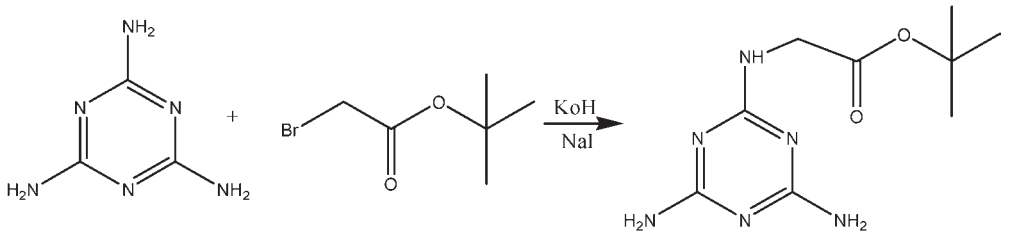
Preparation of Coating Antigen MEL−BSA and Immunogen MEL−Thyroglobulin (TG). The coating antigen MEL−BSA was synthesized as indicated below. Ten milligrams of MEL hapten, 40 mg of EDC, and 15 mg of NHS were dissolved in 1 mL of DMF. The mixture was stirred for 24 h to get solution I. Twenty milligrams of BSA was completely dissolved in 3.0 mL of PBS (pH 8.2) to get solution II (hapten/BSA = 26:1, molar ratio). Then, solution I was slowly added into solution II and stirred at room temperature for 5 h. After that, the mixture was dialyzed against 0.01 mol L^{-1} PBS for 3 days, with dialyzing medium regularly changed twice a day to remove free hapten. Finally, the mixture was centrifugated at 7000*g* for 30 min. The supernatant was collected to get coating antigen MEL−BSA. Then the coating antigen was stored at −20 °C for future use. The immunogen MEL−TG was synthesized in the same way, except that 25 mg of TG was used in the preparation of solution II.

Immunization, Cell Fusion, and Purification of MAbs. BALC/c mice were immunized intraperitoneally with 150 μg of MEL−TG conjugate in an equal volume of cFA (*14*). Mice were immunized for a total of three times at 2 week intervals with the same amount of MEL−TG in iFA. A booster injection was performed using the same amount of MEL−TG in iFA. After the immune response was validated, the splenocytes from immunized mice were fused with logarithmically growing hypoxanthine−aminopterin−thymidine (HAT)-sensitive mouse myeloma cells Sp2/0 (9:1) according to the polyethylene glycol (PEG) method (*15*). The culture supernatants were screened by indirect ELISA described below and cloned by limited dilution method (*16*). The hybridoma cells were cultured in the medium (pH 7.4) containing 0.2% $NaHCO_3$ and RPMI 1640 in 20% newborn calf serum at 37 °C to obtain MAbs for MEL. The purification was performed by acid−ammonium sulfate method, and the purified MAbs obtained were stored at −20 °C.

Indirect Competitive ELISA (cELISA) Procedure. The MEL concentration was determined by the indirect competitive ELISA, which is indicated as follows: 96-well polystyrene microtiter plates were coated with 100 μL/well of 0.25 μg mL^{-1} MEL−BSA solution dissolved in coating buffer overnight at 4 °C. The plate was then washed twice with 280 μL/well of washing buffer and blocked with 200 μL/well of blocking buffer by incubation for 2 h at 37 °C. After that, blocking buffer was removed, and the plate was washed again. A series of diluted analyte solutions (MEL standard solution or MEL containing food samples, 50 μL/well) were added, then MEL MAbs solution (50 μL) was added, followed by 30 min of incubation. The plate was washed as described before and further incubated with 100 μL/well of goat anti-mouse HRP at 37 °C for 30 min. Substrate solutions A and B were added in turn, and the plates were incubated for another 30 min at 37 °C. Color development was stopped by adding stopping solution, and absorbance for each well was determined at 450 nm. The serum taken before immunization was used as a negative control. The inhibition percentage is given as follows: % inhibition = $\%B/B_0$, where B is the absorbance of the well containing competitor and B_0 is the absorbance of the well without competitor. IC_{50} is the competitor concentration resulting in an absorbance value that was half of B_0.

Standard Curve Generation. The MEL−BSA (0.20 μg mL^{-1}) was used as a coating antigen, and indirect cELISA was carried out as described above. MEL solution, 0, 2.5, 5, 15, 45, 135, and 405 μg L^{-1} (PBS, pH 6.6), was analyzed by the immunoassay developed. The B/B_0

Scheme I



Scheme II

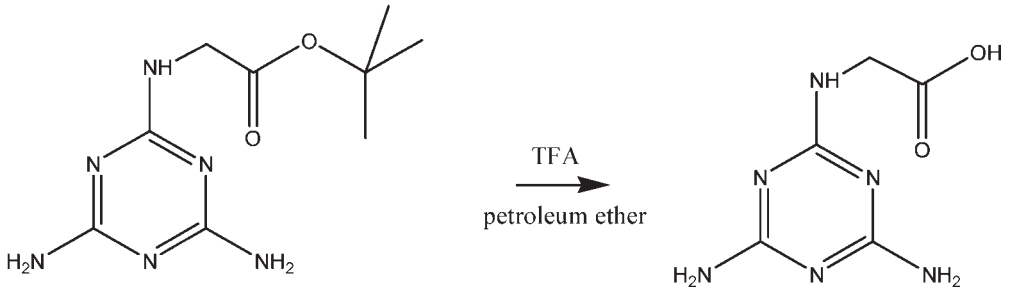


Figure 2. Synthesis procedure for MEL hapten.

generated was plotted versus the logarithmic concentration of MEL to get an inhibition curve.

Antibody Specificity. Specificity of the antibody was monitored by detection of cross-reactivity toward structurally related compounds to MEL by the cELISA test, including cyanurate, ammelide, and ammeline. The IC_{50} value for each compound was determined according to its inhibition curve. Cross-reactivity (CR%) values were calculated according to the following equation: $CR\% = IC_{50,\ MEL}/IC_{50,compounds} \times 100$.

Matrix Effect Determination. Raw milk, milk powder, and animal feeds were used to evaluate the efficiency of the cELISA test to quantify MEL in various matrices. The sample treatment procedure of raw milk, milk powder, and animal feeds was performed as described below. For raw milk without heat treatment, 100 μL of raw milk was diluted with 900 μL of reconstituted solution for analysis. For milk powder, 1 g of milk powder was diluted with 5 mL of 0.02 M acetate buffer. The mixture was then centrifuged at 1000*g* for 5 min. After that, 100 μL of supernatant was mixed with 900 μL of reconstituted solution for analysis. For animal feeds, 1 g of animal feeds was extracted by 10 mL of acetonitrile. The solution was then centrifuged at 1000*g* for 5 min. One milliliter of supernatant was dried on a water bath at 45 °C. One milliliter of *n*-hexane was added, and the mixture was shaken. Then 1 mL of reconstituted solution was added. The *n*-hexane in supernatant was removed by centrifugation at 1000*g* for 5 min. One hundred microliters of aqueous phase in the lower layer was diluted with 900 μL of reconstituted solution for analysis. For all three matrices, 50 μL of the reconstituted samples was used for analysis.

The three matrices were spiked by MEL. The final MEL concentrations were as follows: 0.5, 1.0, and 2.0 mg L^{-1} in milk; 1.0, 2.5, and 3.0 mg kg^{-1} in milk powder; 1.5, 2.0, and 3.0 mg L^{-1} in animal feeds. The samples were then treated as described above. The prepared matrix samples were analyzed to estimate the accuracy, repeatability, and sensitivity of the test. The detection limit for MEL in matrix was calculated by mean observed MEL concentration in 20 samples with no MEL added plus 3 times the standard deviation. Intra-assay variation was determined by analyzing one extraction sample four times on a single day. Interassay variation was determined by analyzing one extraction sample on three different days.

Validation of the ELISA Method by LC-MS Analysis. To validate the immunoassay, MEL-spiked raw milk was determined by LC-MS system with an Agela ASB C18 column (250 × 4.6 mm, 5 μm, Agela Technologies, Tianjin, China). The column temperature was set to be 40 °C. Mobile phase A was prepared by dissolving 1.0 g of octane sulfonate, 2.75 g of NaH_2PO_4, and 2.0 g of citric acid in 1000 mL of H_2O. The mobile phase applied was a mixture of mobile phases A and B (methanol) at a ratio of 70:30 (v/v). Flow rate was 1.0 mL min^{-1}.

For preparation of samples, 2.0 g of raw milk was mixed with 5 mL of methanol and 5 mL of 1% TFA. The mixture was then centrifuged at 1000*g* for 5 min. Six milliliters of the supernatant was mixed with 2 mL of 1% TFA and 5 mL of *n*-hexane. The mixture was centrifuged again to collect 6 mL of clear solution in the lower phase for clean-up. The clean-up procedure was carried out by MCX column solid phase extraction (Waters, Japan). The column was activated by elution with 5 mL of methanol and 5 mL of H_2O in turn. Then, the sample was loaded and eluted with 5 mL of H_2O, 5 mL of methanol, and 5 mL of 5% ammonia (methanol) in turn. The eluate was dried and completely dissolved in 1 mL of methanol/H_2O (2:8, v/v). Then, 20 μL aliquots of the solution were injected into the LC-MS system and detected at 240 nm. The peak area of MEL was recorded, and the concentration was calculated from the standard curve obtained.

RESULTS AND DISCUSSION

Preparation of Conjugates. To connect with thyroglobulin to be antigenic, MEL was designed to react with *tert*-butyl bromoacetate to get MEL hapten (2-*N*-carboxymethyl-1,3,5-triazine-2,4,6-triamine; *N*-carboxymethylmelamine). The MEL hapten was coupled with thyroglobulin according to the carbodiimide method. The molar ratio of MEL hapten to thyroglobulin was optimized as 26:1 according to antibody titer result. The ratio with the highest antibody titer obtained was selected to prepare immunogen. To confirm the structure, the MEL hapten was determined by LC-MS (**Figure 3**). The mass result (185 $[M + H]^+$, 183 $[M - H]^-$) is in good accordance with the molecular weight (MW) of the hapten ($C_5H_8O_2N_6$, MW = 184.07), demonstrating the successful acquisition for MEL hapten.

Characterization of the Assay. The IC_{50} value, inhibition curve, and specificity were investigated for characterization of the assay. The IC_{50} value was used to express the sensitivity of the cELISA method we developed. The representive competitive inhibition curve from 10 to 90% inhibition (absorbance value in the range of 0.126−0.978) was obtained and is shown in **Figure 4**. The IC_{50} value was 22.6 ± 1.9 μg L^{-1}, whereas the LOD, defined as the IC_{10} value from the inhibition curve (**Figure 4**), was approximately 2.5 μg L^{-1}. Compared with the most sensitive commercial ELISA kit for MEL, the Abraxis Melamine Plate Kit (IC_{50} = 100 μg L^{-1}, LOD = 9 μg L^{-1} for MEL in PBS), the test developed in

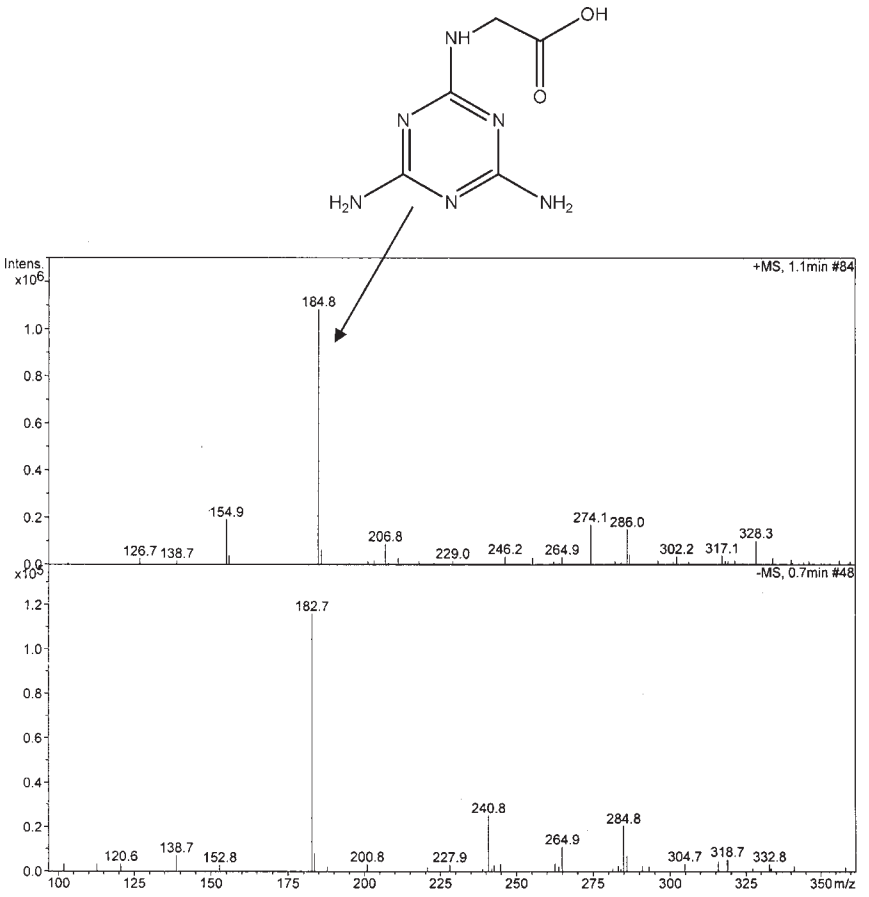


Figure 3. MS profile of melamine hapten.

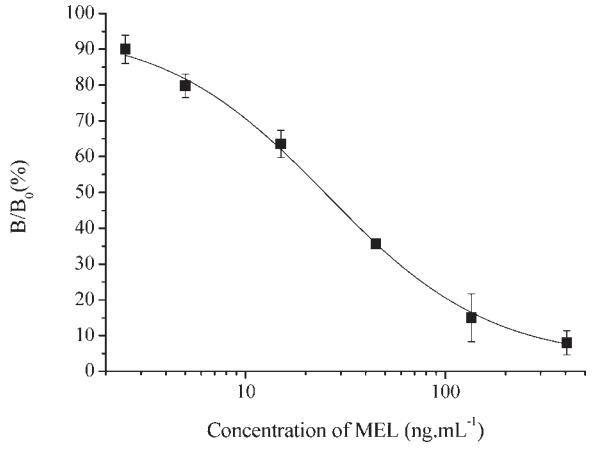


Figure 4. Competitive inhibition curve of MEL (n = 5).

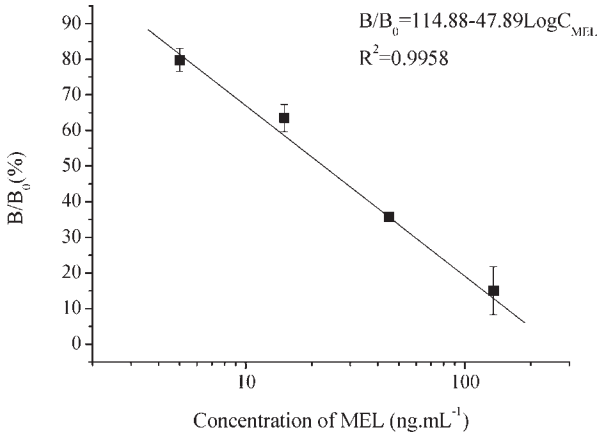


Figure 5. Standard calibration curve of indirect competitive ELISA for determination of MEL in buffer solution.

our research is more sensitive. The B/B_0 value with 10−80% inhibition of maximal absorbance value was plotted versus the logarithmic concentration of MEL to obtain a standard calibration curve for calcuating MEL concentration in buffer solution (**Figure 5**).

Because cyanurate, ammelide, and ammeline are main hydrolysis products for MEL, the specificity of MAbs was evaluated by measuring inhibition curves using the four structurally related compounds (MEL, cyanurate, ammelide, and ammeline) as competitors (**Figure 1**). The selection of tautomers for these compounds was arbitrary, and the cross-reactivity studies were carried out by adding various free competitors with different concentrations to get their respective IC_{50} value and then compare this value with that of MEL. IC_{50} values and cross-reactivities for each compound are given in **Table 1**. The result showed that cyanurate, ammelide, and ammeline exhibited low cross-reactivity (≤1%) toward the antibody, indicating high specificity of the antibody we prepared. These results suggest that the three NH_2 groups in the triazine ring are an important structural factor to determine the affinity. Besides cyanurate, ammelide, and ammeline, triazine herbicides are also structurally related to MEL. They mainly present in cereals and vegetables. Considering the different source for contamination, cross-reactivity of the method with triazine hercides was not tested.

Determination of MEL in Raw Milk, Milk Powder, and Animal Feeds. Matrix effect was estimated in this research, using three types of matrix systems, including raw milk, milk powder, and

Table 1. Cross-Reactivity of Anti-melamine Antibody toward Selected Compounds

compound	cross-reactivity (%)
melamine	100
cyanurate	1
ammelide	<1
ammeline	<1

Table 2. Inter- and Intra-assay Variation of Raw Milk, Milk Powder, and Feed Spiked with Melamine

sample	level	recovery, % (n = 4)	intra-assay variation,[a] % (n = 4)	interassay variation,[b] % (n = 3)
raw milk	0.5 mg L^{-1}	102.6	9.9	11.8
	1.0 mg L^{-1}	110.6	11.5	12.3
	2.0 mg L^{-1}	93.4	9.2	11.4
milk powder	1.0 mg kg^{-1}	101.4	8.5	9.7
	2.5 mg kg^{-1}	103.7	9.8	10.2
	3.0 mg kg^{-1}	98.5	10.3	10.9
feeds	1.5 mg L^{-1}	92.0	8.3	8.3
	2.0 mg L^{-1}	85.4	7.3	7.2
	3.0 mg L^{-1}	79.9	7.2	7.0

[a] Intra-assay variation was determined by analyzing one extraction sample four times on a single day. [b] Interassay variation was determined by analyzing one extraction sample on three different days.

animal feeds. For matrix preparation, raw milk was treated by dilution; milk powder was prepared by centrifugation to get the completely dissolved solution before dilution; feed was mixed with acetonitrile to extract MEL, and the supernatant containing MEL was skimmed by using *n*-hexane before dilution. To minimize the matrix effect, the dilution factor (1:2, 1:5, 1:9) for sample preparation was optimized in the preliminary study. Results showed that when the food sample with no MEL addition was diluted at 1:9, the absorbance value was almost the same as that for the buffer group. Therefore, the dilution factor of 1:9 was selected for sample treatment.

The three matrix samples were spiked with different levels of MEL to determine recovery, intra-assay variation, and interassay variation. The recovery ratios were calculated by the standard calibration curve (**Figure 5**). The results are summarized in **Table 2**. It can be seen that the cELISA gives satisfactory results with recovery ratios in the range of 79.0−111.0%. The intra-assay coefficient of variation was < 12.0% and the interassay coefficient of variation was < 13.0%, which complied with specification issued by the Ministry of Agriculture of the People's Republic of China (Document [2005]17). In addition, the detection limit was estimated to be 0.1 mg L^{-1} for milk, 0.2 mg kg^{-1} for milk powder, and 0.5 mg L^{-1} for feeds.

For further characterization of the method, 30 commonly used animal feeds were selected from a local food company or market and analyzed by the test, including cat food, dog food, meat bone powder, rapeseed cake, fish meal, mixed feed, premixed feed, complete feed, and bean pulp. The detection limit and criteria for acceptance were 0.5 and 2.5 mg kg^{-1}, respectively. As is shown in **Table 3**, the cELISA method could be successfully used as a screening method to detect MEL in most animal feeds as low as 0.5 mg kg^{-1}, which is 20 times lower than the FDA minimum reporting level of 10 mg kg^{-1}.

Validation of the ELISA Method with LC-MS Analysis. To evaluate the quality of cELISA developed in our research, LC-MS was applied to detect the raw milk sample spiked with MEL. As is indicated in **Figure 6**, MEL could be detected at the retention

Table 3. Application of the cELISA Method To Quantify the MEL Residue in Animal Feeds from a Local Food Company and Market

no.	sample	resource	MEL detected, mg L^{-1}	results	acceptable?[a]
1	cat food 1	food company	0.0	nd[b]	yes
2	cat food 2	food company	0.090	nd	yes
3	cat food 3	food market	1.788		yes
4	cat food 4	food market	0.292	nd	yes
5	dog food 1	food company	0.390	nd	yes
6	dog food 2	food company	0.415	nd	yes
7	meat bone powder	food market	0.156	nd	yes
8	rapeseed cake 1	food market	0.396	nd	yes
9	rapeseed cake 2	food company	0.356	nd	yes
10	fish meal 3	food company	0.321	nd	yes
11	fish meal 4	food company	0.230	nd	yes
12	fish meal 5	food company	0.473	nd	yes
13	fish meal 6	food market	33.916*		no
14	mixed feed 1	food market	0.283	nd	yes
15	mixed feed 2	food market	0.217	nd	yes
16	mixed feed 3	food market	0.611		yes
17	mixed feed 4	food company	0.513		yes
18	mixed feed 5	food company	0.409	nd	yes
19	mixed feed 6	food company	4.770*		no
20	mixed feed 7	food company	0.421	nd	yes
21	mixed feed 8	food company	0.415	nd	yes
22	mixed feed 9	food company	0.250	nd	yes
23	mixed feed 10	food company	9.167*		no
24	complete feed 1	food market	0.097	nd	yes
25	complete feed 2	food market	0.002	nd	yes
26	complete feed 3	food company	0.002	nd	yes
27	premixed feed 1	food market	4.206*		no
28	premixed feed 2	food market	0.330	nd	yes
29	corn feed	food market	0.301	nd	yes
30	bean pulp	food company	0.366	nd	yes

[a] When the detected MEL level is >2.5 mg L^{-1}, the sample is not acceptable. [b] nd, not detectable.

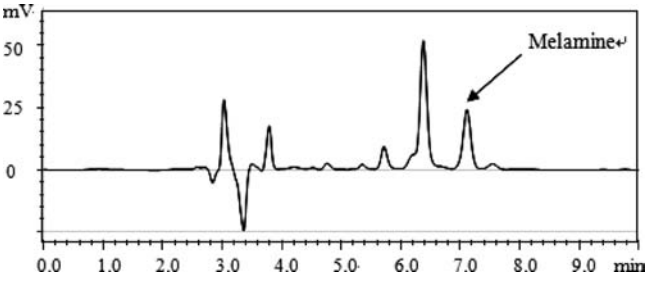


Figure 6. LC-MS analysis of raw milk spiked with 2 mg L^{-1} melamine.

time of 7.2 min. The linear relationship of peak area (A) with MEL concentration (C) in buffer was $C = 0.0123A - 30.5$ (R^2 = 0.9999), with the linear range from 0.5 to 2.0 mg L^{-1}. By this equation, the raw milk spiked with 0.5, 1.0, and 2.0 mg L^{-1} of MEL was determined, and the detected MEL concentration was calculated accordingly. As is shown in **Figure 7**, the results from these two methods correlated very well, indicating the reliability of the test we developed. Moreover, the test showed a higher recovery than LC-MS at the three levels. We speculated that the chromatographic purification procedure used in the sample treatment might be the reason for the loss of MEL in LC-MS. Compared with the cELISA, LC-MS analysis was more stable, suggested by the lower standard deviation.

In summary, immunogen and coating antigen of MEL were successfully prepared, and monoclonal antibody was also developed. An indirect cELISA method was accordingly established to detect MEL in the range of 5.0−405.0 μg L^{-1}, with the IC_{50} value of 22.6 ± 1.9 μg L^{-1}. The MAbs showed high specificity toward MEL. The raw milk, milk powder, and animal feeds spiked with

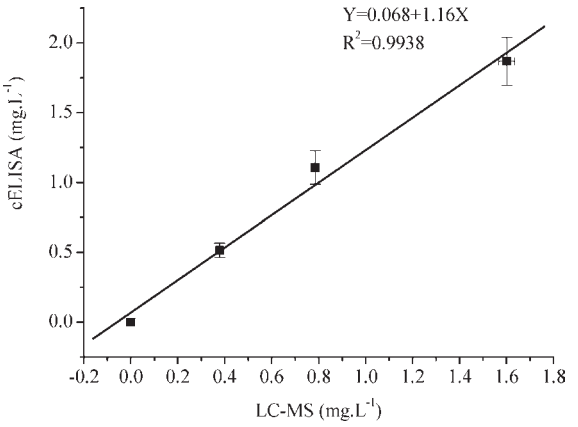


Figure 7. Correlation of cELISA versus LC-MS analysis for raw milk spiked with 0.5, 1.0, and 2.0 mg L^{-1} melamine ($n = 3$).

MEL were evaluated, and excellent results have been obtained. The detection limits were 0.1 mg L^{-1} for milk, 0.2 mg kg^{-1} for milk powder, and 0.5 mg L^{-1} for feeds, much lower than the 1 mg L^{-1} limit set by most countries. The recovery ratio was 79−110%. The intra-assay and interassay coefficients of variation were < 12.0 and $< 13.0\%$, respectively. The suitability of the cELISA method to screen and quantitate MEL in most kinds of feeds from local markets was also verified.

ABBREVIATIONS USED

BSA, bovine serum albumin; cBSA, cationized BSA; cFA, complete Freund's adjuvant; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; IC_{50}, concentration at 50% inhibition; iFA, incomplete Freund's adjuvant; MEL, melamine; NHS, *N*-hydroxysuccinimide; OVA, ovalbumin; PBS, phosphate-buffered saline; SAS, saturated ammonium sulfate.

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