



## Research Paper

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# Rapid Enrichment and High-sensitivity Detection of Melamine from Milk with Immunomagnetic Microspheres

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## ABSTRACT

One of the key concerns of the people and the government of China is food safety. After the use of melamine as an additive was reported in 2008, melamine detection was required in milk. The current methods to detect and confirm melamine are mainly HPLC and HPLC-MS/MS, but the pre-treatment processes include some tedious steps making them time-consuming and environmentally unfriendly. In contrast, the on-line detection of melamine utilizing colloidal gold test paper was appreciated, but its sensitivity has not yet complied with the demands. Here, we report a novel and rapid method for enriching the melamine content of milk using immuno-magnetic microspheres, which may allow the 100-fold concentration of melamine within 20 min. The combination of these methods allowed a decreased detection time, an improved detection efficiency of LC-MS/MS and improved the sensitivity of on-line melamine detection, helping milk processing factories to detect melamine in their products.

**Key words:** Melamine, enrichment, immuno-magnetic microspheres, LC-MS/MS, colloidal gold test paper.

## INTRODUCTION

Dairy products are indispensable components of a healthy diet for all age groups due to their high nutritional value (Hilding-Ohlsson et al., 2012). Melamine (2, 4, 6-triamino-1, 3, 5-triazine, MA) is usually used in the production of melamine-formaldehyde resin. In plastics manufacturing, resins are necessary for the production of surface coatings, laminates, adhesives, and flame retardants (Sugita et al., 1990). The use of melamine-formaldehyde resins as coatings that come into contact with food was approved by the US Food and Drug Administration only when the yield of chloroform-soluble extractives in the food contact surface does not exceed 0.5 mg/in<sup>2</sup> (21 CFR 177.1460). Because the protein content of milk is important for the production of many products and significantly impacts their sensory and rheological characteristics, this metric has thus been used as a measure of quality by many industries (Hilding-Ohlsson et al., 2012; Sanvido et al., 2010).

Melamine is strictly forbidden to use as an additive in food or related ingredients, and melamine-contaminated pet food ingredients in the USA were reported to cause renal failure and death in cats and dogs in 2007 (Liu et al., 2010). Although, the problem of adulteration with melamine was recently alleviated, some studies indicated that the presence of melamine in our daily lives may be due to many other sources, such as food packaging materials and pesticide breakdown (Lutter et al., 2011; Zheng et al., 2012). Since melamine is a nitrogen-rich compound, it can be illegally added to animal feed or protein materials to fraudulently increase the apparent protein content.

In 2007, thousands of pet illnesses and deaths in America proved to be caused by melamine-contaminated pet food. In 2008, the continuous consumption of melamine-contaminated milk and infant milk powder resulted in renal stones in thousands of Chinese children. Because melamine usually exists in low levels, it is important to determine the

melamine content sensitively and accurately (Zhang et al., 2014). Therefore, it is important to monitor residues of melamine in edible products. To date, many papers reported the use of HPLC (Ehling et al., 2007; Sun et al., 2010; Wei et al., 2009), LC/MS (Andersen et al., 2008; Turnipseed et al., 2008), GC/MS (Zhu et al., 2009) and other methods (Yu et al., 2009) for determining the melamine content in Chinese cabbage, chard, protein sources, pet food, meat, fish, soil and various matrixes. Although, these methods provide both qualitative and quantitative analyses, they are time-consuming and require expensive instruments compared with ELISA, which is a low-cost, portable and sensitive method capable of screening a lot of samples. ELISA may thus be a practical tool for the routine monitoring of melamine residues in animal-derived products. The development of an ELISA method for determining the melamine content has been reported, and the commercial ELISA kits have been used to detect melamine in pet food (Garber et al., 2008; Kim et al., 2008). Liu et al. (2010) developed a simple and sensitive ELISA method for simultaneous determination of the melamine content in animal meat tissues based on a polyclonal antibody specific to melamine. Furthermore, the ELISA results were confirmed by HPLC.

Since the introduction of encapsulated magnetic materials in the 1960s, a wide variety of magnetic microspheres (MMS) has been applied to the immuno-magnetic separation (IMS) of target proteins (Chang, 1966). To date, magnetic carriers based on proteins immobilized onto MMS have been extensively used in enzyme oxidation, medical immunoassays, and affinity purification (Cao et al., 2007).

In this study, a cellulose binding domain-protein A (CBD-ProA) was expressed and used as a critical component in the development of magnetic cellulose microspheres (MCMs) -antibody conjugates. The MCMs were activated using a melamine antibody conjugated through CBD-ProA and were utilized to separate melamine from milk. After enrichment and elution, the melamine-containing eluent was analyzed by LC-MS/MS. The sensitivity was increased 50-fold within 20 min in comparison with that achieved by other methods. Thus, it may be possible to use this approach in combination with colloidal gold test paper for the on-line detection of melamine, and improving the detection sensitivity 100-fold.

## MATERIALS AND METHODS

*Escherichia coli* strain BL21 (DE3) /pET-35b (+) -ProA, which can express the fusion protein CBD-Protein A, was constructed as previously reported (Cao et al., 2007). Bovine serum albumin (BSA), diaminobenzidine (DAB), *o*-phenylenediamine (OPD), and horseradish peroxidase (HRP) -labeled goat anti-mouse IgG were purchased from Junyao Co., Ltd. (Tianjin, China). Mouse anti-melamine monoclonal antibodies (mAb) were supplied by Fapon

Biotech Inc. (Shenzhen, China). All of the other reagents were of analytical grade and commercially available.

## Preparation of Magnetic Cellulose Microspheres (MCMs)

MCMs were prepared using a suspension-embedding procedure based on the optimized method reported by Tang et al. (2010). The mixture of viscose solution and ferro fluid powder (10%, v/w) was well dispersed using a homogenizer (FA25, Fluco, England) and added to the reactor and churned at 16,000 rpm for 5 min. The solution was stirred for 2 h at 70°C to solidify the liquid particles into microspheres. After cooling to room temperature, batches of the prepared MCMs were isolated.

## Characterization of Magnetic Microspheres

The morphological characterization of the microspheres was performed through scanning electron microscopy (SEM X-650, Hitachi, Japan). The size distribution of the microspheres was determined using a laser granulometer (Mastersizer 2000, Malvern, UK).

## Activation of MCMs

The recombinant CBD-ProA fusion protein was over expressed to immobilize antibodies on the MCMs surface. After culturing and induction, the crude extract containing CBD-ProA was obtained from *E. coli* BL21 (DE3) /pET35b (+) -ProA and analyzed by western blotting (Ausubel et al., 1995). For immobilization of CBD-ProA, highly homogenous distribution of MCM particles with good spherical geometry was prepared using the suspension-embedding procedure and added to the crude extract. The mixture was then incubated at room temperature for 30 min. The MCMs were separated using a magnetic separator and washed with PBS until the protein could no longer be detected in the supernatant. A mouse mAb against melamine was then mixed with protein A-CBD-MCM at ambient temperature for an additional 15 min. Any excess protein was removed and calculated using a BCA kit, and the IgG binding capacity of the MCMs calculated through a modified ELISA method using activated MCMs instead of 96-well plates (Bai et al., 1997) (n=3). HRP-labeled goat anti-mouse IgG (1:2000 dilution) was added to the MCMs. After incubation, the activity of bound HRP was measured at 490 nm using an automatic ELISA analyzer with OPD as a substrate.

## Melamine enrichment

The separation efficiency of the bio-conjugated MCMs was

**Table 1.** Quantitative ions\*, qualitative ions and related parameters.

Analyte	Quantitative ions (m/z)	Cone voltage (V)	Collision voltages (v)	Retention Time (min)
Melamine	127.1/68.1	35v	25v	1.91
	127.1/85.1*	35v	17v	

determined using the microspheres to capture melamine from milk samples. The capture procedure was performed according to the following methods: First, samples of approximately 100 ml of milk were concentrated by centrifugation at 5000 rpm and 4°C for 10 min. Purified melamine was then added to the milk samples to final concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 ng/ml respectively. Then, 20- $\mu$ L aliquots of activated MCMs were added to the samples, and the samples were then incubated at room temperature for 15 min and separated using a magnetic separator. After washing with PBS, 1 ml of elution buffer (Gly-HCl, 0.1 M, pH 2.5) was added, and the samples incubated for 5 min with a slight stirring at room temperature. Finally, the supernatant was gathered and filtered using ultrafiltration membranes (MW3500) for further analysis. The aforementioned procedure was replicated thrice.

### Analysis of melamine with LC-MS/MS

LC-MS/MS was used to measure the melamine content. Chromatographic separation was achieved using a 100-mm  $\times$  2.1 mm ACQUITY UPLC BEH HILIC analytical column with a 1.7  $\mu$ m particle size (Waters, USA). The mobile phase was a gradient prepared from Milli-Q-water containing 0.1% formic acid and acetonitrile (95% v/v) linearly changed as follows: 0 min, 95%; 4 min, 85%; 4.1 to 6 min, 95%. The flow rate was 0.3 ml/min, the injection volume for both the standards and the sample solutions was 10  $\mu$ L, and the column temperature was 30°C. A tandem quadrupole mass spectrometer (Waters Quattro Premier XE) equipped with an ion spray interface was used with positive ion detection and operated in the multiple reaction monitoring mode (MRM). Nitrogen (purity grade of 5.0) was used as the collision gas, drying and nebulizing gas. The optimized ESI(+)-MS-MS operating conditions were as follows: capillary voltage, 3.5 kV; source temperature, 110°C; desolvation temperature, 400°C; desolvation gas flow rate, 600 L/h; and cone gas flow rate, 50 L/h. The quantitative ions, qualitative ions, and related parameters are shown in Table 1.

Calibration curves were constructed using blank samples spiked with melamine at concentrations ranging from 5 to 100 ng/ml to assay the linearity of the method (n=3). The calibration curves were achieved by a linear regression analysis of the peak area versus the concentration, and these exhibited good linearity and a linear correlation

coefficient greater than 0.99. After capture by melamine mAb-coated MCMs, the supernatants were analyzed. The analytical recovery (%) of each group was calculated as follows:  $\text{recovery (\%)} = \frac{\text{concentration measured}}{\text{concentration fortified}} \times 100$ .

### Determination of melamine content using colloidal gold test paper

The enrichment method as earlier described was combined with colloidal gold test paper to detect melamine in enriched spiked and actual samples. After the samples and test papers had been restored to room temperature, 200  $\mu$ L of each sample was dropped onto the test paper and incubated at room temperature for 5 min. The color change of the colloidal gold test paper in the test and control groups was observed.

## RESULTS AND DISCUSSION

### Preparation and characterization of magnetic cellulose matrices

To apply suitable immuno-magnetic cellulose matrices to efficient affinity separation applications, the optimized conditions were used according to the method of Tang et al. (2010). As shown in Figure 1A, regular beads could be observed by SEM. A mean diameter of 10.3  $\mu$ m was determined using a laser granulometer, and the yield was 85.9% (Figure 1B).

### Activation of MCMs

After expression, western blotting was used to detect the CBD-ProA fusion protein. Compared with controls (Figure 2A, lines 1 and 3), an obvious band was observed in the extract from cells expressing CBD-ProA (Figure 2A, line 2). After incubation in the crude extract, the MCMs were carefully washed and detected by modified ELISA, and a positive signal was then observed (data not shown). The melamine mAb was added, and after incubation, the supernatant was measured using a BCA kit. Almost no protein was detected at a lower initial concentration of melamine mAb, whereas, approximately 10 mg/ml protein was measured at a higher initial concentration (20 mg/ml);

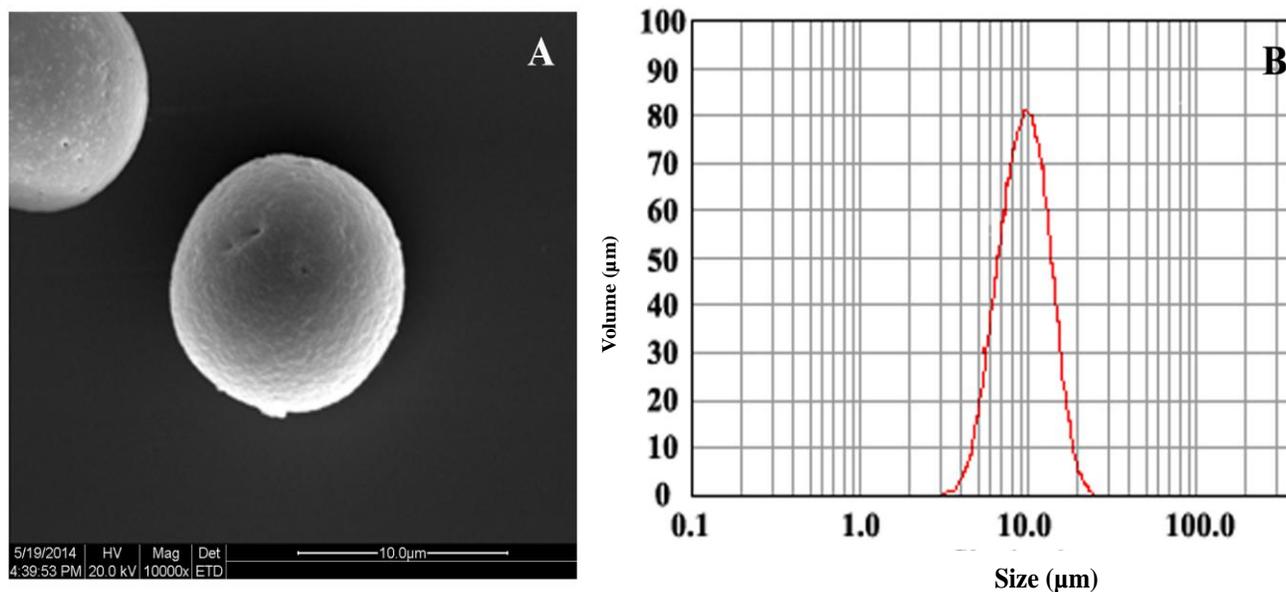


Figure 1. SEM (A) and diameter analysis (B) of magnetic cellulose beads

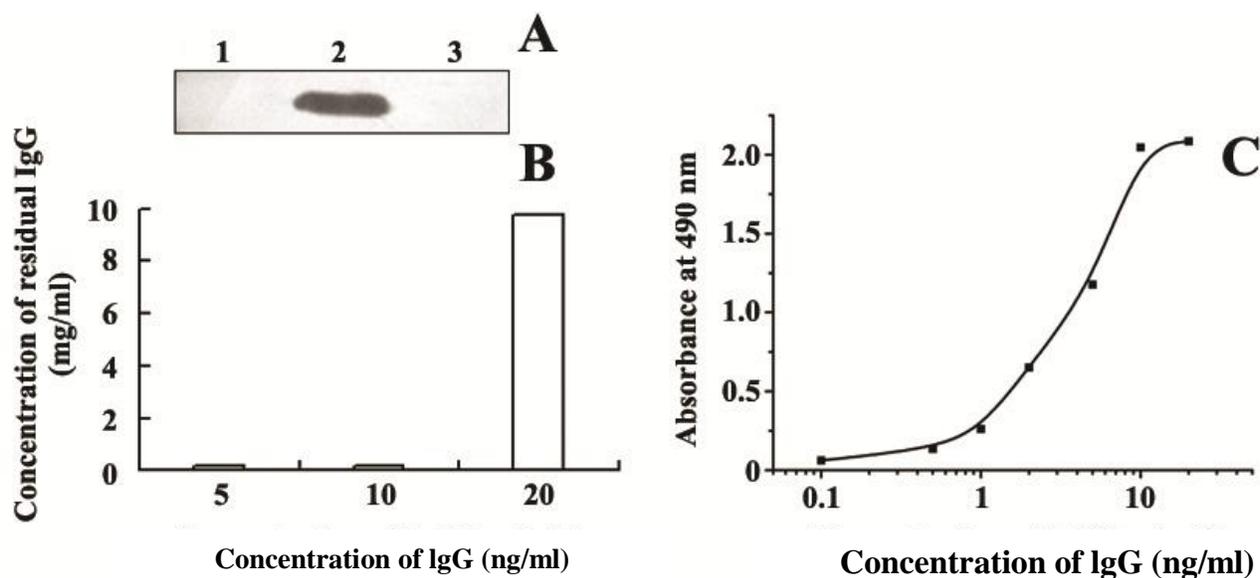


Figure 2. Expression of CBD-ProA fusion protein and activation of MCMs A: Western blot of the CBD-ProA fusion protein; 1: *E. coli* BL21(DE3) without plasmid; 2: *E. coli* BL21(DE3) with pET35b(+)-ProA; 3: *E. coli* BL21(DE3) with pET354b(+). B: BCA of residual IgG. C: Analysis of absorbed IgG by modified ELISA.

Figure 2B). The ELISA method was then used to measure the coated IgG further on the MCMs. As shown in Figure 2C, the addition of 10 mg/ml IgG resulted in peak signal intensity at 490 nm. This increase was not observed at the initial concentration of IgG. These two results both showed that the adsorption of IgG by our MCMs was approximately 10 mg/ml.

### Analysis of melamine

LC/MS was used to characterize melamine. After isolation by HPLC, the characteristic productions for melamine were selected and detected using multiple reaction modes. The retention time of melamine was 1.91 min (Figure 3A), and a standard curve for melamine was generated by analyzing

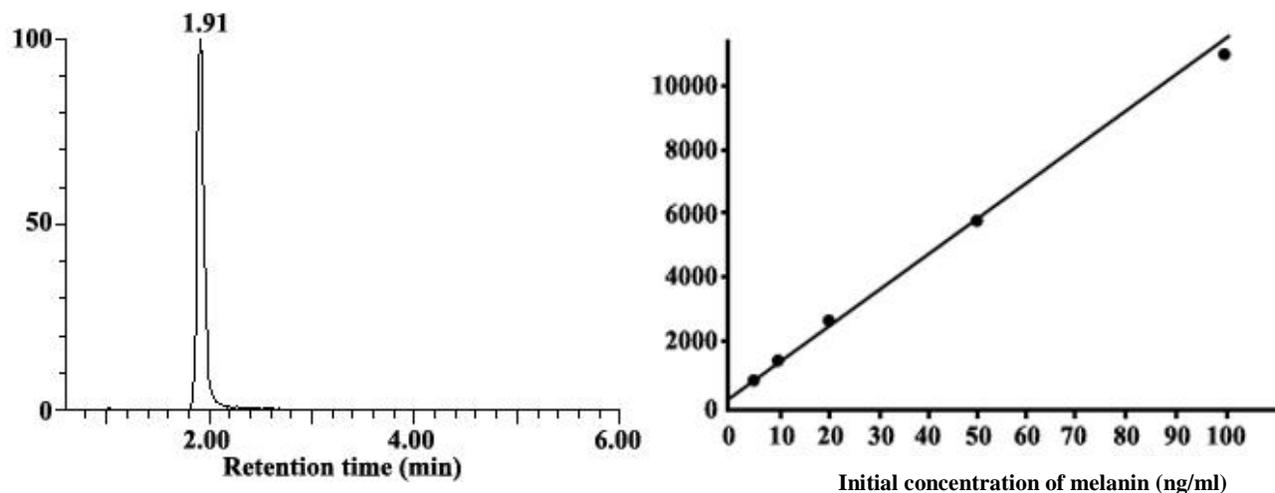


Figure 3. (A) LC-MS/MS analysis of melamine and (B) standard curve of melamine.

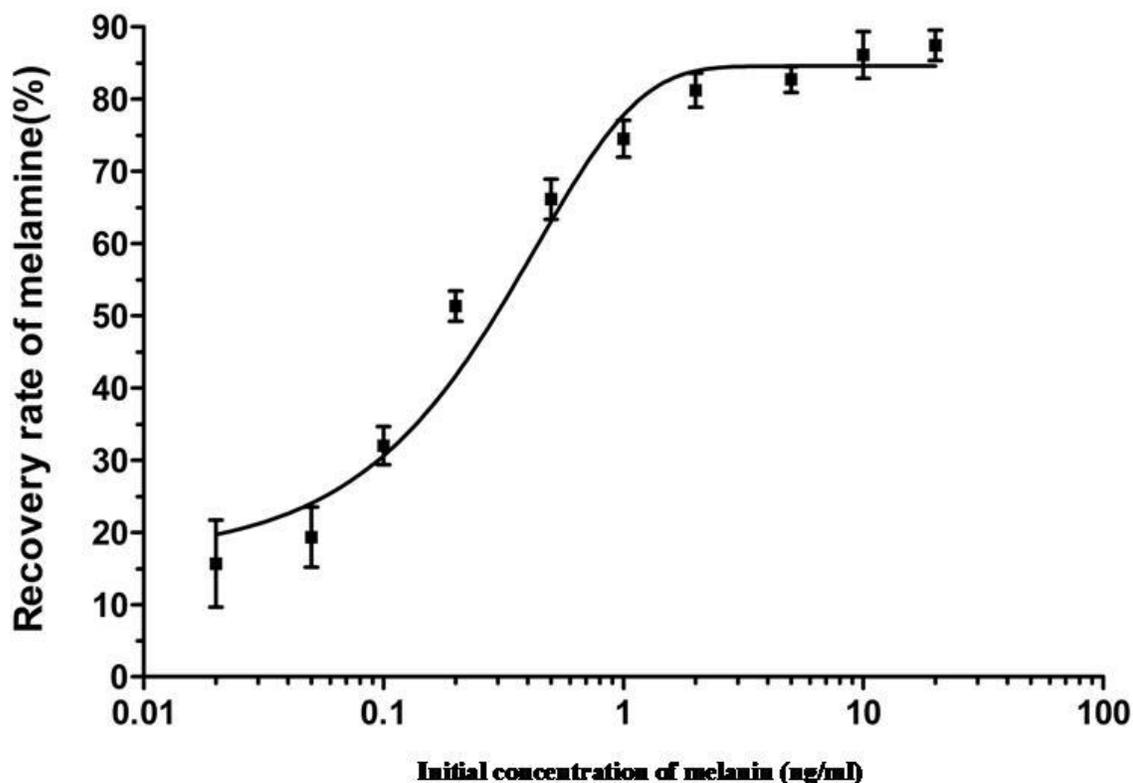


Figure 4. Detection curve of melamine by LC-MS/MS after enrichment.

melamine-negative milk spiked in duplicate with a standard solution of melamine at concentrations of 0, 5, 10, 20, 50, and 100 ng/ml respectively. The standard curve was drawn according to the peak area (Figure 3B). The calibration curve was  $y = 128.1x + 334.19$ , and the correlation coefficient 0.9988.

Using an established method, the enrichment of melamine

from the milk samples was extensively studied. The amount of melamine recovered by activated IgG-ProA-CBD-MCMS beads was determined through LC-MS/MS. As shown in Figure 4, when the initial concentration of melamine was 2 ng/ml, melamine could be efficiently enriched, and the mean recovery rate was higher than 80% ( $n = 3$ ). At reduced concentrations, the mean recovery rate decreased.

When the recovery rate was higher than 50%, the result was considered credible and reliable. The detection limit was 0.2 ng/ml. These results indicated that the enrichment method improved the detection sensitivity by at least 50-fold (GB/T22388-2008, 2008; Pan et al., 2013). All of the concentrations were analyzed using colloidal gold test paper with the enrichment processing method. The results showed that 0.5 ng/ml melamine could be detected efficiently after enrichment. A weakly positive detection of 0.2 ng/ml melamine was observed, which was at least 100-fold higher in sensitivity than the results obtained from the direct use of colloidal gold test paper (data not shown).

## Conclusions

This work introduced the use of CBD-ProA to immobilize antibodies to MCMs, which can create effective affinity chromatography matrices for the separation of small-molecular-weight compounds from food samples. In this study, IgG-ProA-CBD-MCMs were prepared using a simple procedure, and after optimization, these microspheres were readily adsorbed and melamine separated from milk samples. The bi-specific fusion protein CBD-ProA displayed superior attributes in the linkage between MCMs and antibodies as compared with conventional chemical modifications. The use of IgG-ProA-CBD-MCMs allowed the efficient and convenient isolation of small molecules and compounds from complex food samples. This represents a significant improvement over previously available techniques. The detection sensitivity was improved by at least 50-fold as compared with the classical method and more than 100-fold compared with the direct use of colloidal gold test paper.

Preliminary studies indicated that this simple and economical method may be further developed for use in food safety testing.

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