Analytical Methods

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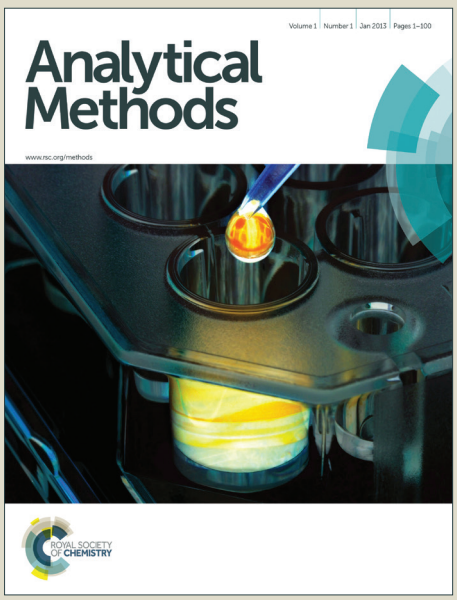

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Paper

A simple, highly sensitive, high throughput and organic solvent-free screening method for melamine by microsphere-based flow cytometry immunoassay

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Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxooooox

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In recent years the safety problem of infant formula powder has provoked panic among Chinese citizens. One of the most notorious incidents was the 2008 Chinese milk scandal which harmed thousands of infants. Development of a fast, high throughput screening method towards melamine and other harmful chemicals is of paramount importance. Microsphere-based flow cytometry is a new multiplexing immunochemical method for food-testing. Through competition between free analyte in sample and analyte-coupled microspheres, the content of analyte can be indirectly quantified by measuring fluorescence emitted from labelled antibodies. This developed method of high sensitivity (Limit of detection = 0.70ng/ml) has already been validated and successfully applied to screen for melamine in various brands of infant formula powder analysis fulfilling the legislated tolerance level in a simple, fast, high throughput and organic solvent-free manner.

1. Introduction

2,4,6-triamino-1,3,5-triazine, or more famously known as melamine, is one of the most commonly used industrial chemicals in various manufacturing processes, such as the production of melamine resins tableware, coatings, commercial filters, glues or adhesives, fertilizers and flame retardant. [1-2] It is not allowed to be added into foods or food products, but melamine food contamination is possible through the use of melamine resin tableware, or as a metabolite product of cyromazine due to the application of this insecticide on animals or crops. [1] Apart from being an environmental contaminant, more seriously is its potential abuse as an adulterant in infant formula milk powder to mimic the presence of high protein levels. The 2008 Chinese milk scandal finally led to occurrence of kidney stones in thousands of infants, and at least six deaths were reported. [3] After this incident, many countries have adopted the acceptable level of 1mg/kg for infant formula milk powder and 2.5μg/ml for other foods containing more than 15% milk as recommended by World Health Organization. [4] Public confidence towards formula milk powder from China is still weak even several years after that incident. [5] Therefore the development of effective melamine detection method is of paramount importance for regulatory units for public health as well as a popular research area among analytical scientists. [4, 6, 7] Among those developed methods, chromatographic techniques coupled with mass spectrometer are the most popular analytical methods to be employed, owing to their strong separation and confirmation power. [8, 9] However, these techniques usually requires complex sample pre-treatment, clean-up or even derivatization steps. The running time is also long, which highly restricts their usefulness in high-throughput analysis. A simple, quick, and cost-effective way that can act as a preliminary screening method to reduce suspicious samples for further confirmatory MS-based techniques analysis is much desired.

Microsphere-based flow cytometry has become a popular method in recent years for food monitoring purpose. [10-13] By using different classes of microspheres that can be differentiated by their different ratios of two internal fluorophores, the Luminex 200 system is able to distinguish up to 100 different microspheres by a red laser. The system then uses a green laser to quantify fluorescent trackers that are attached on each microsphere. Multiplexing can be easily achieved simply by incubating sample with several classes of microsphere to allow rapid quantitation of multi-analytes. [14] There

is a more recent advance in assay manipulation procedures that uses carboxylated microsphere with magnetic property which allows an easier washing step with higher recovery of microspheres. [15, 16] We herein first report on microsphere-based flow cytometry immunoassay for detection of melamine. A melamine derivative was coated onto the carboxylated surface of microsphere by covalent bonding. The coated beads were then employed for detection of melamine in infant formula milk powders through a competitive inhibition immunoassay format. In this format, with more melamine in the sample solution for competition, fewer monoclonal primary antibodies could be captured by the microsphere-bound melamine derivative. Therefore less secondary antibody, which was labelled with fluorescent reporter molecule phycoerythrin, could bind to the attached primary antibody in the later step. Melamine content in samples could be indirectly quantified from the extent of fluorescence quenching. The developed method was validated to show the capability for real samples analysis according to the legislated tolerance level set in Hong Kong; it also demonstrated superior advantages of being simple, high throughput, fast and organic solvent-free (except synthesis part) when compared with other melamine detection methods.

2. Materials and methods

2.1 Chemicals, reagents and materials

Melamine was obtained from Meryer Chemical Technology Ltd. Co. (Shanghai, China). Atrazine and ammelide were obtained from TCI (Shanghai, China). Ammeline was obtained from Lancaster Synthesis (Massachusetts, USA). Cyanuric chloride, ethylenediamine, sodium chloride, sodium hydroxide, sodium phosphate monobasic, sodium phosphate dibasic, potassium chloride, Tween-20, Bovine serum albumin (BSA), cyromazine, N-hydroxysuccinimide (NHS) and 1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich (Hong Kong, China). Melamine-Monoclonal antibody was obtained from Creative Diagnostics (New York, USA). Phycoerythrin Goat Anti-Mouse Ig was purchased from SantaCruz Biotechnology (Hong Kong, China). Magnetic carboxylated microsphere (MC10026-01), sheath fluid, 96-wells flat bottom plates, amine coupling kit, calibration and verification kit, handheld magnetic washer were obtained from Biorad (Hong Kong, China). Magnetic Particle Separator was obtained from Invitrogen (Hong Kong, China). A 96-wells plate shaker was obtained from IKA (Hong Kong, China). Refrigerated centrifuge 5417R was obtained from Eppendorf (Hong Kong, China). A melamine-ELISA kit was obtained from Romer Labs (Redhill, Singapore). Ammonium hydroxide solution (25%) and hydrochloride acid were from reagent grade commercial sources. 0.1M PBS was prepared by weighing 80g sodium chloride, 12.8g sodium phosphate monobasic and 4.5g sodium phosphate dibasic dehydrate into 1000mL deionized water with pH tuned to 7.4. 0.01M PBS was prepared by dilution from 0.1M PBS with pH tuned to 7.4. PBSBT was 0.01M PBS supplemented with 0.1% (w/v) BSA and Tween-20 respectively. N^2–(2-aminoethyl)-1,3,5-triazine-2,4,6-triamine

2.2 Synthesis of coating antigen

The synthesis of coating antigen is a two-steps reaction as shown below:

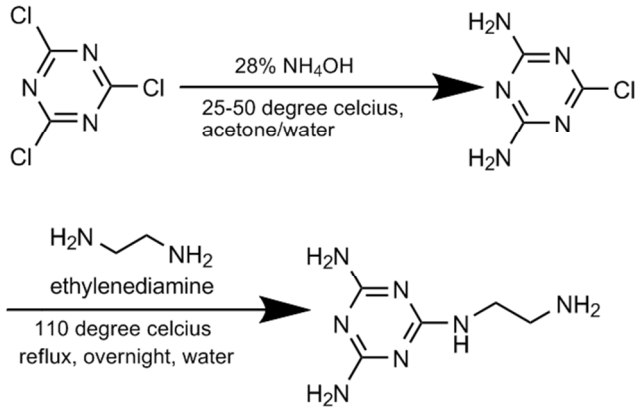


Figure 1. Two-steps reaction scheme for the synthesis of AETT

Cyanuric chloride (17.9g) was first dissolved in 100ml acetone. 100ml water was then added to the solution and cooled to 0˚C. 32ml 25% aqueous ammonium hydroxide solution was added dropwise. The solution was then warmed to 25 ˚C and stirred overnight. The mixture was filtered and washed with 30ml THF three times. The white solid was then dried by vacuum. (13.2g, 93.1%) ^{1}H NMR (400MHz, DMSO-d6): δ (ppm) 7.104-7.177(doublet), ^{13}C NMR (100MHz, DMSO-d6): δ (ppm) 167.47, 169.11

After obtaining CAAT, AETT was synthesized from it by Zhou's method [17]. Briefly, ethylenediamine (3.1g) was dissolved in water, followed by addition of CAAT (1.45g). The resulting mixture was heated to reflux at 110˚C overnight. The reaction mixture was cooled in an ice bath, and any insoluble white solids were filtered off. The filtrate was concentrated by vacuum to yield a yellow oily mixture

which was distilled to obtain a yellow solid. The yellow solid was then washed with 40ml ethanol three times to obtain a white solid, which was further dried by vacuum. (1.00g, 30%) ^{1}H NMR (400MHz, DMSO-d6): δ (ppm) 2.905 (t, 1H), 3.37 (q, 4H), 6.164-6.306 (doublet, 4H), 6.690 (t, 1H), 8.181(b, 2H) ^{13}C NMR (100MHz, D_2O): δ (ppm) 37.30, 39.54, 165.90, 166.23. ESI-MS found intense m/z at 170 which corresponds to $[M+H]^+$.

2.3 Immobilization of coating antigen onto microspheres

AETT was immobilized onto carboxylated microspheres by NHS/EDC conjugation chemistry as suggested by the protocol of amine coupling kit using BSA as a linker. [18-19] Briefly, $5x10^5$ microspheres were washed and activated by 10µl 50mg/ml NHS and EDC for 20 min at room temperature in darkness. Then 100µg BSA was incubated with the activated microspheres for 2 hours at room temperature. After coupling of BSA, the carboxyl groups of the coupled BSA were activated by NHS and EDC at pH 6, and incubated with 100µg AETT overnight. Afterwards, any unreacted carboxyl groups were blocked by incubating with blocking buffer (PBS-TBN: PBS containing 0.1% BSA, 0.02% Tween-20 and 0.05% sodium azide, pH 7.4), and then stored in storage buffer at 4˚C in darkness. All the incubation steps were performed in Eppendorf tubes with constant shaking, while the washing steps were performed using a magnetic particle separator. The final microsphere concentration was determined by a hemocytometer.

2.4 Preparation of infant formula milk powder

0.2g infant formula powder and 1g sodium chloride were weighed in a centrifuge tube. 10ml of 0.1M PBS was added, vortexed for 10 seconds and then shaken for 10 min to dissolve the powder and salt. After that, the mixture was centrifuged at 13,000 rpm at room temperature for 10 min. The clear fraction in the middle was ready for immunoassay detection.

2.5 Preparation of melamine standards

In this report, due to the complexity of matrices in infant formula milk powder, a matrix-match calibration was needed to overcome the effect. For those chosen formula milk powders of different brands, after they had been tested by a commercial ELISA to confirm the absence of melamine, they were pooled together in equal portions and prepared according to the above procedure. The prepared solution from pooled powders was used for preparing a series of melamine standards by dilution from a stock melamine solution (1000µg/ml), which was prepared by dissolving 0.05g melamine in 50mL 0.01M PBS.

2.6 Competitive inhibitory immunoassay for melamine detection

A competitive inhibition type immunoassay was designed to detect melamine in samples. (See Figure 2) AETT on the microsphere surface and melamine in samples would compete for free monoclonal anti-melamine antibodies (primary antibody). After removing any unbound molecules, phycoerythrin-linked goat anti-mouse Ig (secondary antibody) would be added to provide fluorescence signals to those bound primary antibody. Melamine concentration in sample can be deduced indirectly from the fluorescence signals.

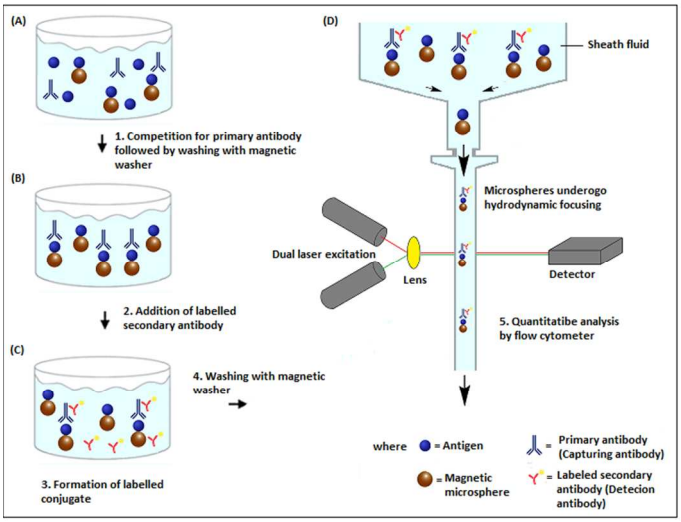


Figure 2. Schematic diagram of microsphere-based competitive inhibitory typed - flow cytometry immunoassay

The detection was performed in the wells of a 96-wells flat bottom plate. First, 2000 microspheres were added to each well. The supernatant solution was removed using a handheld magnetic washer. Then 100µl of melamine standards or sample solution was added to the well. 50µl of the primary antibody (0.58µg/ml) prepared in 0.01M PBS was added subsequently and the mixture was incubated for 1 hour. Afterwards the supernatant solution was removed and washed with 150µl PBSBT three times. A volume of 50µl secondary antibody (3µg/ml) prepared in 0.01M PBS was then added and incubated for 30 min. Then each well was washed with 150µl PBSBT three times, and reconstituted with 125µl PBSBT for

measurement. All the incubation steps were performed at room temperature and in darkness with constant shaking (750rpm).

2.7 Instrumentation and data analysis

The fluorophore linked to the secondary antibody and microsphere was quantified using a Luminex 200 analyzer. A red laser (635nm) was used to classify microsphere, while a green laser (532nm) was used to excite phycoerythrin. The emission was then detected at 565-585nm. The doublet discriminator gate (DD gate) was optimized and set at 6300-22000. The number of minimum bead count was set as 100. The sample acquisition volume was set as 75µl (See Supp. Info. Figures 1 & 2 for typical results obtained). Generally measurement of each well would take less than one minute. The calibration points were fitted using GraphPad Prism 6.0 by the Logistic 5PL equation set in Bio-Plex Software Manager (Brendan Scientific, Carlsbad, CA, USA) by a non-linear regression fitting procedure:

$$y = d + \frac{a - d}{[1 + \left(\frac{x}{c}\right)^{b}]^{g}}$$

where x is the concentration, y is the response, a is the estimated response at zero concentration, b is the slope of the tangent at midpoint, c is the midrange concentration or midpoint, d is the estimated response at infinite concentration, and g is an asymmetry factor. [20]

For every experiment all conditions were tested at least in duplicate, and the results are expressed as the mean value ± SEM (standard error of the mean).

3. Results

3.1 Optimization of working conditions

Optimization was performed with respect to different parameters to look for the most sensitive working conditions before spike analysis:

3.1.1 Amount of coating antigen for immobilization

Three concentrations of AETT (10, 50 and 100µg) were tested to see if the sensitivity of the method would be affected. The calibration plots for these three batches of microspheres were plotted and compared (See Supp. Info. Figure 3). Basically the three curves showed no significant differences to each other. They showed similar sensitivity, since they nearly overlapped when expressed in percentage inhibition versus concentration curve, and their only difference was in their median fluorescence intensity. With higher concentration of AETT for immobilization, the median fluorescence intensity was higher under the same conditions. Therefore the highest tested concentration of AETT (100µg) was chosen to achieve a higher resolution for that method.

3.1.2 Competition format

Both one-step and two-steps incubation with coupled microsphere with coupled microsphere, primary antibody and sample solution have been reported in literature. [10-11, 14, 21-22] In this study, three competitive formats were compared and the corresponding calibration curves were plotted to show their effects. (See Supp. Info. Figure 4) As observed, for the third format (green curve), sensitivity was the poorest when the primary antibody was incubated with microsphere first. For the first and second formats (blue and red curves respectively), again the calibration curves nearly overlapped when expressed in percentage inhibition curve, meaning they had equal sensitivity. But when comparing their median fluorescence intensity, the first format gave higher intensity and thus higher resolution. Besides, since one-step incubation was also simpler and more convenient to perform, the first format was chosen.

3.1.3 Incubation time with the primary antibody

The incubation time with the primary antibody is one of the most significant factors to determine the extent of competition between microsphere and melamine in sample, hence it greatly affect the sensitivity of the method. Five different incubation durations (15, 30, 60, 90, 120min) were tested and compared. Generally, as the incubation time shortened from 120 to 15 min, the calibration curve shifted to the left, which means it is more suitable for low concentration analysis. (See Supp. Info. Figure 5) But the compensation compromise was a smaller median fluorescence intensity, which led to poorer resolution and precision. This could be explained by not having enough time for reaching equilibrium. As the method is designed to be a fast and high throughput screening method, the middle incubation duration, 60 min, was chosen. This incubation time was reasonably fast, and the observed precision was quite good along its dynamic range. Moreover, the time delay effect that occurs when handling large batch samples analysis could be minimized by employing this incubation time.

3.1.4 Antibody concentration

The two factors that we need to make a balance when optimizing antibody concentration are fluorescence intensity and cost. For the primary antibody, fluorescence levelled off as the concentration increased to up to around 0.58µg/ml, so this level was employed for further tests. (See Supp. Info. Figure 6) For the secondary antibody, as the concentration increased from 50 to 3000 ng/ml, the median fluorescence intensity measured was higher, since more secondary antibodies had been successfully bound to the microsphere (See Supp. Info. Figure 7). Fluorescence still showed an increasing tread in this testing range. Higher concentrations were not tested due to the higher costs they would incur and the highest level tested, 3µg/ml, was employed for further tests.

After optimization of the abovementioned working parameters, the finalized calibration graph of melamine with maximized sensitivity was obtained (See Figure 3). $IC_{50,}$ which defined the sensitivity of the assay, was found to be 7.58 ng/ml, while IC_{10} - IC_{90}, which defined the dynamic range of the method, were from 1.01 ng/ml to 94.30 ng/ml. [14] The limit of detection was calculated by subtracting three times the standard deviation of median fluorescence intensity in the absence of melamine (n=16) from the maximum fluorescence signal of the curve. The LOD was calculated to be 0.70 ng/ml. The method exhibited high sensitivity towards melamine when compared with other melamine detection methods.[4, 6-7]

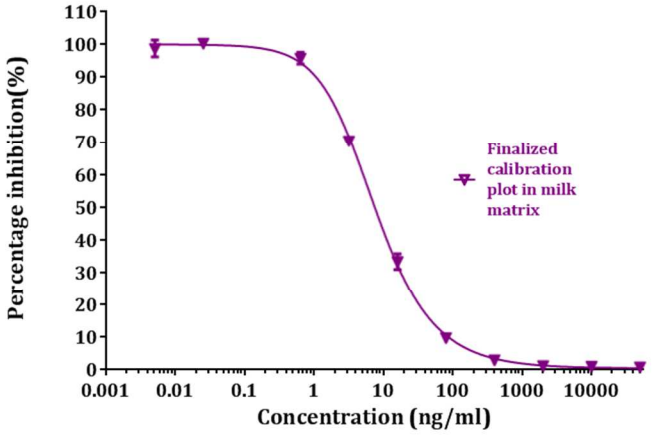
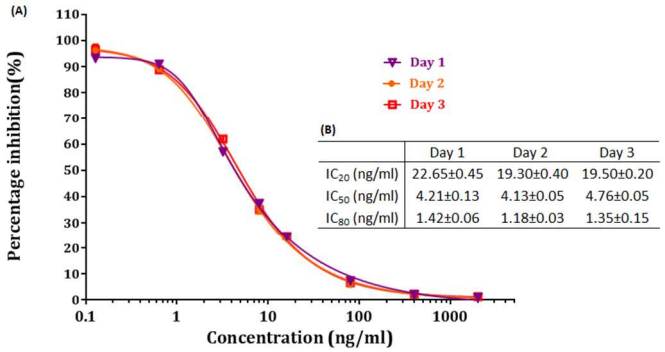


Figure 3. Finalized calibration curve of melamine in milk matrix

3.2 In-house validation of developed assay

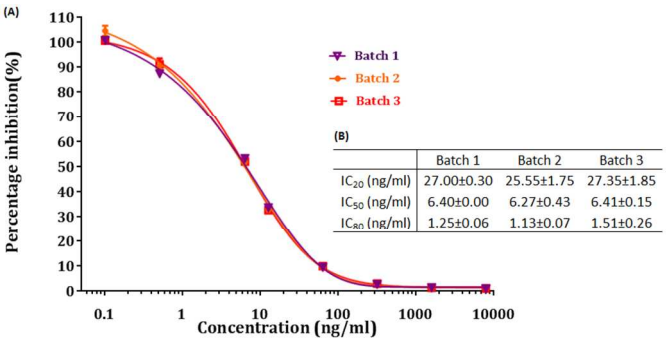
3.2.1 Reproducibility

The calibration curves using either the same batch of immobilized microspheres or three independent batches of immobilized microspheres were plotted on different days and compared (See Figures 4 and 5). IC_{20}, IC_{50} and IC_{80} were used to compare the shape of the calibration curves to see if they were significantly different to each other. Results of Student's t-test showed that there were not any significant differences for the three trials in either inter-batch or intra-batch reproducibility. ($p < 0.05$)



	Day 1	Day 2	Day 3
IC_{20} (ng/ml)	22.65±0.45	19.30±0.40	19.50±0.20
IC_{50} (ng/ml)	4.21±0.13	4.13±0.05	4.76±0.05
IC_{80} (ng/ml)	1.42±0.06	1.18±0.03	1.35±0.15

Figure 4. (A) Calibration curves of melamine on different days using the same batch of immobilized microspheres under the same conditions (Range: 0.128 – 2000ng/ml). Each data point was expressed as the percentage inhibition in the absence of melamine. (mean ± SEM, n=2) (B) IC_{20}, IC_{50} and IC_{80} of the corresponding three calibration curves. (mean ± SEM, n=2)



	Batch 1	Batch 2	Batch 3
IC_{20} (ng/ml)	27.00±0.30	25.55±1.75	27.35±1.85
IC_{50} (ng/ml)	6.40±0.00	6.27±0.43	6.41±0.15
IC_{80} (ng/ml)	1.25±0.06	1.13±0.07	1.51±0.26

Figure 5. (A) Calibration curves of melamine using three independent batches of immobilized microspheres under the same conditions (Range: 0.128 – 10000ng/ml). Each data point was expressed as the percentage inhibition in the absence of melamine. (mean ± SEM, n=2) (B) IC_{20}, IC_{50} and IC_{80} of the corresponding three calibration curves. (mean ± SEM, n=2)

3.2.2 *Specificity*

To test for the specificity of the method, four compounds structurally similar to melamine (See Figure 6) were chosen and the corresponding calibration graphs were plotted using the same procedures (See Figure 7). The cross-reactivity values were calculated according to the equation:

$$CR\% = IC_{50\ of\ melamine} / IC_{50\ of\ compounds} \times 100$$

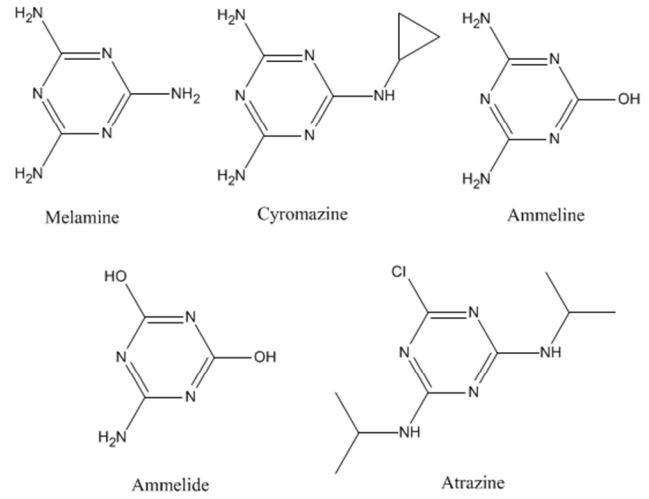


Figure 6. Chemical structures of melamine and four structurally similar compounds

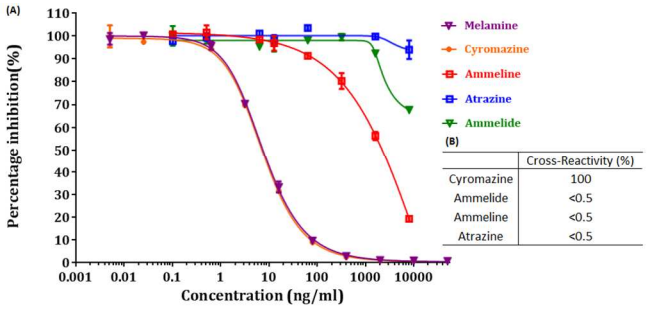


Figure 7. (A) Calibration curves of melamine, cyromazine, ammeline, atrazine and ammelide. Each data point was expressed as the percentage inhibition in the absence of that compound. (mean ± SEM, n=2) (B) Cross-reactivity values to melamine

3.2.3 *Comparison of spike analysis to commercial ELISA kit*

The spike recoveries of melamine at four concentration levels (0.1, 0.5, 1.5 and 10 μg/g) were fortified into six brands of infant formula powders, which had been tested by a commercial ELISA confirming the absence of any melamine, and then measured by both the developed method and a commercial ELISA kit. The results are summarized in Table 1:

Infant formula powder	Concentration fortified (μg/g)	Mean recovery(%) obtained from microsphere-based method	RSD(%)	Mean recovery(%) obtained from a commercial ELISA kit	RSD(%)
Brand 1	0.1	102.5	2.13	Nil (out of detection range)	
	0.5	102.0	0.26	124.6	15.99
	1.5	97.8	5.94	83.5	5.09
	10	122.6	4.60	111.2	5.43
Brand 2	0.1	120.3	5.88	Nil (out of detection range)	
	0.5	117.8	2.67	110.2	5.79
	1.5	103.7	3.41	80.6	24.61
	10	111.6	4.85	86.1	12.81
Brand 3	0.1	99.5	3.30	Nil (out of detection range)	
	0.5	91.7	2.34	127.3	9.81
	1.5	94.5	2.18	79.9	5.15
	10	102.3	2.10	127.4	5.85
Brand 4	0.1	77.7	7.41	Nil (out of detection range)	
	0.5	89.2	1.10	134.3	6.11
	1.5	97.2	2.72	80.9	5.85
	10	89.8	7.18	115.9	7.90
Brand 5	0.1	105.5	2.84	Nil (out of detection range)	
	0.5	120.0	2.30	120.8	0.44
	1.5	115.7	7.81	80.4	16.51
	10	123.1	6.23	116.8	7.31
Brand 6	0.1	109.5	3.95	Nil (out of detection range)	
	0.5	127.0	2.50	170.2	9.88
	1.5	108.3	6.57	88.3	12.02
	10	123.1	5.70	125.3	15.01

Table 1. Recovery of melamine from spiked samples determined by the developed microsphere-based method and a commercial ELISA kit (n=3)

4 Discussions

When designing the detection scheme of the method during the early stage of research, AETT was proposed to act as the heterogeneous coating antigen for immobilization onto carboxylated microsphere. The characteristics of this compound are: First it has a high structural similarity to melamine with only one amine group modifying into a spacer arm; the other two amine groups and the triazine ring remain unchanged, and they form the epitope to be recognized by the melamine monoclonal antibody. Second, when many AETT molecules are immobilized onto the microsphere, the ethylenediamine spacer arm can help reduce steric hindrance brought by the bulky triazine ring, and therefore more AETT molecules can be coupled onto the microsphere. Finally the terminal amine group on the spacer arm provides a reactive site for conjugation with carboxyl groups on microspheres through NHS/EDC chemistry. Initially, AETT was immobilized directly onto the microsphere. However, the reproducibility was found to be quite poor when the calibration curve for melamine was plotted (RSDs up to 25%, data not shown). To improve the reproducibility, BSA was employed as a linker between the microsphere and AETT molecules. BSA is a large, water-soluble protein possessing many carboxyl and amine groups. During immobilization, the activated carboxyl groups on

microspheres first reacted with the amine groups on BSA. Afterwards, the carboxyl groups on BSA were activated and reacted with the amine groups on AETT (Two-steps coupling). One thing to pay attention was that the pH should be conducted at pH 6 during the second step of the coupling for preventing self-reaction between carboxyl and amine groups of BSA, which may lead to aggregation of microspheres. [23] Finally the reproducibility improved a lot (RSDs <10%), and it was discovered that BSA actually provided more sites for coupling, which led to stronger fluorescence intensity. When performing the immunoassay assay, in the beginning it was found that the number of beads counted was small (less than 30) even though 2000 beads had been added to each well. The problem, as suggested by A. Meimaridou, was due to the physical adsorption of polystyrene microsphere onto the surface of the well. [15] After changing the washing solvent to PBSBT, the BSA and Tween-20 inside helped minimize the problem and finally the number of beads counted could reach 100.

The samples could not be calibrated by standards prepared in blank solvent due to the complexity of matrices in milk powder (See Figure 8). The two calibration curves differed significantly in median fluorescence intensity, especially at the low concentration region. Therefore matrix-match calibration was essential for an accurate quantitative analysis by preparing standard solutions in the pooled milk matrix. Nevertheless, owing to the different formulas and constituents in each brand of formula powder, the matrix effect in each brand could be quite different due to the different protein contents and properties. Salting out technique was employed by adding sodium chloride during sample preparation to help maintain a similar milk matrix environment. It is an easy and simple pre-treatment method to remove protein in the sample solution without complicating or lengthening the sample treatment procedures. Finally the spike analysis of all the six brands of infant formula powder could give good recoveries in each fortified level.

As abovementioned, to test for the detection ability of the method, six brands of formula powders designed for infants were chosen. A low concentration level (0.1μg/g), high concentration level (10μg/g) and two concentration levels (0.5 and 1.5μg/g) close to the legislated acceptable level (1μg/g) of melamine were fortified into the milk powders. Not only did the developed method give satisfactory spike recoveries, but also the relative standard deviations of all spike levels were all < 8%. When compared with that of an ELISA kit, it showed obvious superiority in terms of sensitivity, precision, accuracy and suitability for analysis of large batch of samples. It also has the potential to be modified into a multiplex method for simultaneous detection of other milk contaminants/adulterants, a feature that that conventional ELISA lacks.

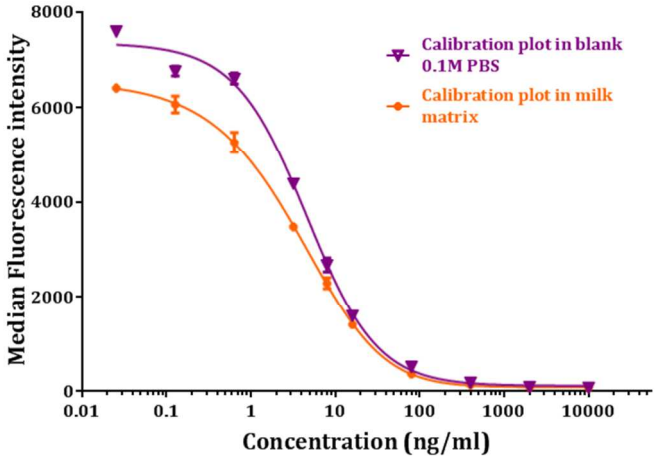


Figure 8. Calibration plot of melamine in blank 0.1M PBS buffer and in milk matrix. Each data point was expressed as mean ± SEM, n=2.

To validate the specificity of the method, cross-reactivity values to other structurally similar compounds were determined. All those compounds exhibited an insignificant cross-reactivity value to melamine (<0.5%), except for cyromazine, which was found to respond exactly the same to the primary antibody as melamine (CR value=100%). From the results we can deduce that the targeted epitope of the chosen primary antibody should consist of two amine groups and one N atom attached to the triazine ring. In this study the monoclonal antibody towards melamine was a limited resource so only one type of commercially available antibody was tested. Other kinds of antibodies need to be tested if higher specificity towards melamine is to be achieved. Cao reported previously a method for the production of a monoclonal antibody which demonstrated a higher specificity towards melamine. [1]

However, the ability to screen for cyromazine should not be viewed as a drawback. For the immunoassay developed in this report, it is designed as a screening tool for food safety purposes. Cyromazine is a triazine pesticide for fly control in cattle manure, field crops, vegetables and fruits by inhibiting insect growth. [25] And melamine is one of its degradation products or metabolite. In 2001, the Chinese Agriculture Department established a Maximum Residue Level (MRL) of 0.05 mg/kg of cyromazine in foods of animal edible tissues. [26] After sample preparation and dilution, the developed method was still capable of screening for it according to the MRL

level established, which means that this method could concurrently detect melamine and cyromazine similar to other previously published literatures. [27-29]

In order to further improve the screening capacity of the assay, polyclonal antibody, which is believed to exhibit a higher sensitivity and wider recognizing ability towards structurally similar compounds, should be more suitable to be employed than monoclonal antibody in developing a screening method.

5 Conclusions

In this report, a simple, high throughput, highly sensitive and organic solvent-free immunoassay towards melamine based on flow cytometry principle was successfully developed. The sensitivity and limit of detection were as low as 7.58 and 0.70 ng/ml respectively. The method was validated and demonstrated good spike recoveries for fortified level ranging from 0.1 to 10μg/ml in six brands of formula powder. It is suitable to be a high-throughput screening, or even quantitative/semi-quantitative tool for melamine in infant formula powder compared to other developed methods, such as ELISA and chromatographic techniques. 96 samples can be analysed in three hours, which can be considered as a fast method. And the property of microsphere-based method allows its further development into a more effective multi-class infant formula contaminants/adulterants screening method.

Acknowledgements

This work was supported by funding from The Hong Kong Polytechnic University.

Notes and references

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Electronic Supplementary Information (ESI) available: [Flow cytometry results and Immunoassay optimization results]. See DOI: 10.1039/b000000x/

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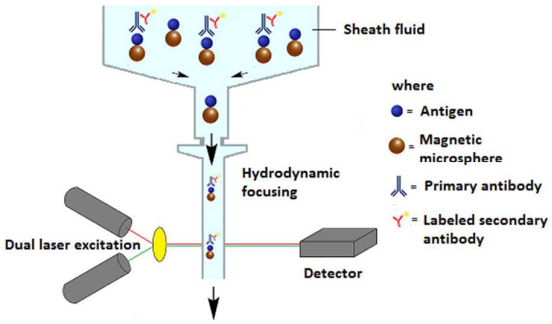

A facile method based on an indirect competitive inhibitory microsphere-based flow cytometry for melamine screening