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A New Method for Determination of Alfatoxin M1 in Milk by Ultrasensitive Time-Resolved Fluoroimmunoassay

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Abstract A competitive indirect time-resolved fluoroimmunoassay (TRFIA) for detecting aflatoxin M1(AFM1) contamination in milk was developed, by using aflatoxin M1-bovineserum albumin conjugate, anti-AFM1 antibody, and Eu-labeled goat anti-rabbit antibody. To improve the sensitivity of the assay, the concentrations of the coating antigen and anti-AFM1 antibody were varied to optimize the condition of the immunological assay. The limit of detection values, limit of quantification values, and dynamic working range were 0.006, 0.022, and 0.022–1.334 μg/kg, respectively. Values of recovery within and between assays were 88.0–116.0% and 92.69–108.63%. The method was applicable for the full-cream, semi-skimmed, skimmed, and raw milk as well. Values of repeatability (intra-laboratory variability) and reproducibility (inter-laboratory variability) were 1.2–4.5% and 0.8–5.0%, respectively. The results of using AFM1-TRFIA to analyze samples of 23 brands of milk that were purchased in Wuxi revealed that AFM1 was absent from all studied samples. This study suggests that the novel method is a simple, sensitive, specific, reproducible, economic, and adequate method for screening large quantities of samples and has good prospects of application.

Keywords Time-resolved fluoroimmunoassay · AFM1 · Europium · Milk

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Introduction

Aflatoxins (AFs) are a class of highly toxic and carcinogenic mycotoxins produced primarily by *Aspergillus flavus*, *Aspergillus parasticus*, and *Aspergillus nomius* (Creppy 2002). These toxins are found worldwide in stage of crop growth, harvest, storage, and processing (Bhat et al. 2010; Caloni et al. 2006; Jiang et al. 2005). AFB1 is the most significant one among all subtypes. By the action of P450 cytochrome enzyme, AFM1 is the hydroxylate metabolite of AFB1 in mammary glands that consume AFB1-contaminated diets (Fallah 2010). It has been classified as class 1 human carcinogen by the International Agency for Research on Cancer (IARC 2002), associating with various of toxicity, including carcinogenesis, teratogenesis, and mutagenesis (Corcuera et al. 2011; Roda et al. 2010). To protect public health, most countries define the maximum acceptable levels of AFM1 in milk and dairy products that vary from 0.05 μg/kg in EU (European Commission 2006) to 0.5 μg/kg in China and in the USA.

Many methods have been established for AFM1 detection previously. High-performance liquid chromatography (HPLC) with fluorimetric detection (FD) (Mao et al. 2015; Muscarella et al. 2007), which successfully superseded the thin-layer chromatography (TLC) (Kamkar 2006), remains one of the most widely used techniques. Currently, liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Chen et al. 2014; Huang et al. 2014) and electrospray ionization quadrupole time of flight mass spectrometry (ESI-CID-MS/MS) (Sirhan et al. 2013) have been developed. All these procedures rely on heavy cost, well-equipped laboratories and a couple of hours, thus impairing their applications in AFM1 detection. On the other hand, enzyme-linked immunosorbent assays (ELISAs) have become popular for AFM1 detection because of low cost and easy application (Peng et al. 2016; Vdovenko et al. 2014). However, ELISA is not stable by using enzyme as biomarker. Also, this

assay is sensitive to assay conditions. It is necessary to establish efficient high-throughput methods to minimize the number of steps and reduce reaction time and errors, getting more reproducible and sensitive results. Therefore, we further integrate a time-resolved fluorescence method for detecting AFM1 in milk. Lanthanide (rare earth) ion chelates showed highly desirable spectral characteristics, including long fluorescence emission life times (Eu^{3+} has a lifetime on the order of millisecond, which is several orders of magnitude longer than the non-specific background) (Karhunen et al. 2011), large stokes shift (200–300 nm) (Binnemans 2009; Ouyang et al. 2011), and high fluorescence intensity. These advantages can reduce the background interference from ubiquitous endogenous fluorenscent components and enable the method stable, sensitive, and reproductible. In recent years, time-resolved fluoroimmunoassay (TRFIA) has been widely used in human diagnostics (Guo et al. 2015; Huang et al. 2006; Zhang et al. 2015) and food safety (Ma et al. 2009; Wang et al. 2016; Zhou et al. 2015).

This paper aimed to develop an extremely sensitive assay for AFM1 by using rare earth ions as labels which was named competitive time-resolved TRFIA. The method was successfully applied to the detection of AFM1 in the sterilized milk without pretreatment, easy to operate, and cost-effective.

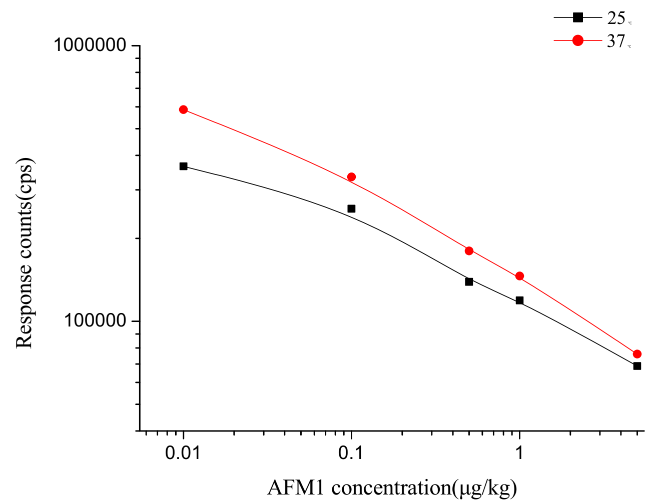

Fig. 2 Standard curve of indirect competitive AFM1-TRFIA at different incubation temperatures

Material and Methods

Reagents and Apparatus

Diethylenetriaminepentaacetate acid (DTPA) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (ST. LOUIS, MO, USA). AFM1-BSA, anti-AFM1 antibody (by immunization with AFM1-KLH), and AFM1-ELISA kit were provided by Chuncheng Zhao (Wuxi, China). Europium-labeled kit including N^1-(p-isothiocy-anatobenzyl)-diethylene-triamine-N^1,N^2,N^3,N^3-tetraacetic acid-Eu chelate (Eu^{3+}-DTTA) was purchased from Perkin Elmer (Turku, Finland). Goat anti-rabbit antibody was purchased from SouthernBiotech (Birmingham, USA). Polystyrene 96-well microtitre plates were purchased from Nunc International (Roskilde, Denmark). β-naphthoyltrifluoroacetone (β-NTA) was synthesized in our laboratory (Hu et al. 2001). Auto DELFIA-1235 fluorometer was purchased from PE-life-science. PD-10 column and Sepharose CL-6B were from Pharmacia (Piscataway, NJ, USA).

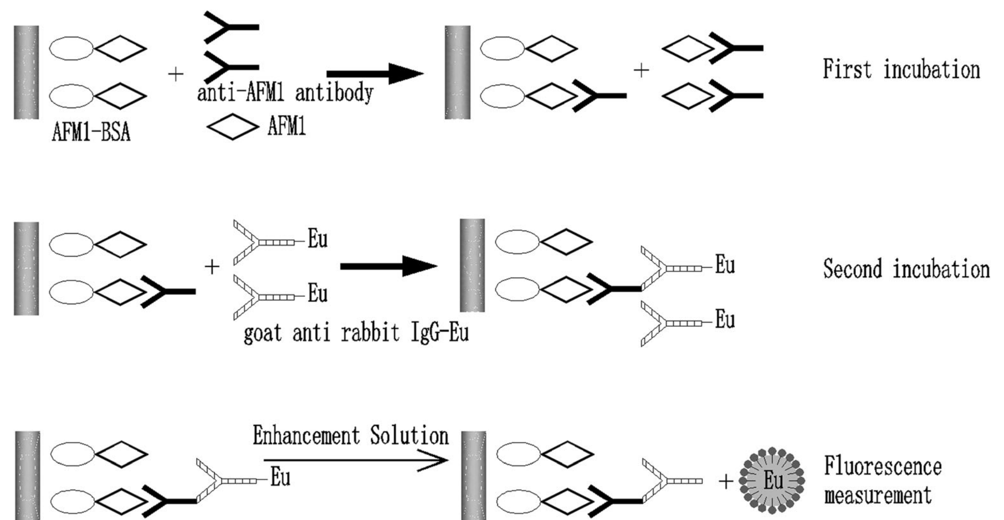

Fig. 1 Scheme of the indirect competitive AFM1-TRFIA for determination of AFM1

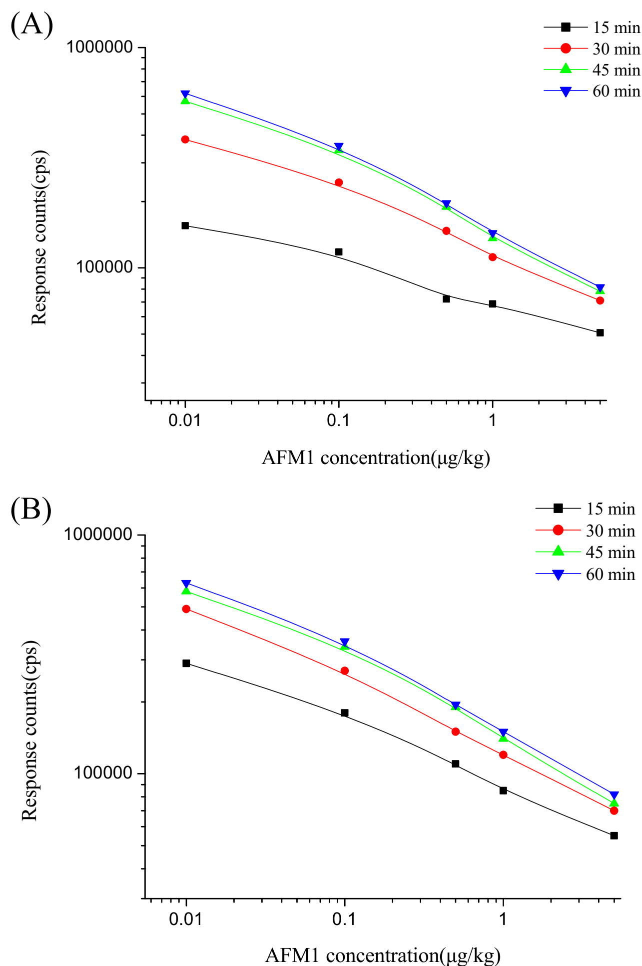

Fig. 3 Standard curve of indirect competitive AFM1-TRFIA at the first incubation step (**a**) and that at the second incubation step (**b**)

Buffer solutions used in the research were coating buffer (0.05 mol/L carbonate-bicarbonate, pH 9.6, containing 0.9% NaCl, and 0.05% sodium azide), assaying buffer (0.05 mol/L Tris-HCl, pH 7.8, containing 0.9% NaCl, 0.2% BSA, 0.01% Tween-20, 20 μmol/L DTPA, and 0.05% sodium azide), washing buffer (0.05 mol/L Tris-HCl, pH 7.8, containing Tween-20, and 0.05% sodium azide), blocking buffer (0.05 mol/L Tris-HCl, pH 7.8, containing 0.9% NaCl, 1% BSA), and PBS buffer (pH 7.4, containing 1.45% $Na_2HPO_4 \cdot 12H_2O$, 0.15% $NaH_2PO_4 \cdot 2H_2O$).

Coating of the Microtitration Wells

The M1-BSA conjugate was dissolved and diluted at concentrations ranging from 1.0 to 0.125 mg/L in coating solution. Then, the polystyrene microtiter wells were coated with 100 μL overnight at 4 °C. The plates were washed twice after immobilization with the washing buffer and blocked with 125 μL/wells of the blocking buffer for 2 h. After blocking, the plates were dried and preserved at −20 °C.

Preparation of Enhancement Solution

The enhancement solution contained 1 ml Triton X-100, 0.1 mol acetic acid, 15 μmol β-NTA, and 50 μmol tri-n-octylphosphine oxide each liter. The working procedures have been described in detail previously (Hu et al. 2001).

Labeling of Antibody with Europium

Goat anti-rabbit antibody labeled with Eu^{3+} was prepared referring to instruction manual. PD-10 column was used to exchange buffers for goat anti-rabbit IgG to pH 8.5 (50 mol/L Na_2CO_3-$NaHCO_3$, containing 0.9% NaCl). The goat anti-rabbit antibody was mixed with Eu^{3+}-DTTA in molar ratio of about 50:1 and the mixture was incubated for 18 h at 30 °C. The labeled antibody was separated and purified by gel filtration on a Sepharose CL-6B column with the elution buffer (pH 7.8, 0.05 mol/L Tris-HCl, containing 0.9% NaCl, and 0.05% sodium azide). The concentration of Eu^{3+} was obtained by fluorescence measurement. The labeled antibody was preserved at −20 °C.

Preparation of Anti-AFM1 Antibody

Two rabbits were immunized by injection of 1 mg AFM1-KLH emulsified in complete Freud's adjuvant per rabbit subcutaneously. After 2 weeks, the second-time injection was

Table 1 Optimization of experimental conditions in competitive step of indirect competitive AFM1-TRFIA

No.	AFM1-BSA/anti-AFM1-pAb (mg L^{-1}/mg L^{-1})	Maximum fluorescence (cps) (n = 3)	IC_{10} (μg/kg) (n = 3)	IC_{50} (μg/kg) (n = 3)	IC_{20}-IC_{80} (μg/kg) (n = 3)
1	0.125/1.0	380,000	0.008	0.219	0.028–1.722
2	0.25/1.0	660,000	0.007	0.196	0.025–1.534
3	0.5/1.0	1,030,000	0.009	0.228	0.031–1.661
4	1.0/1.0	1,470,000	0.020	0.317	0.053–1.883
5	0.25/0.5	360,000	0.006	0.173	0.022–1.334
6	0.25/0.2	190,000	0.010	0.239	0.033–1.739

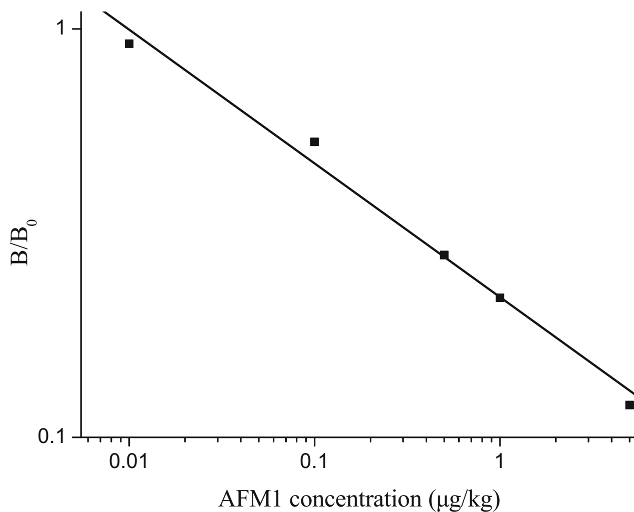

Fig. 4 Standard curve of indirect competitive AFM1–TRFIA

given using the same amount of antigen emulsified in incomplete Freud's adjuvant. Blood was collected from the rabbit's ear vein 7 days after the second injection. The sera was collected by centrifuging at 3000 rpm for 15 min at 4 °C after clotting, and the anti-AFM1 antibody was attained by affinity chromatography.

Indirect Competitive TRFIA of AFM1

A scheme of the indirect competitive AFM1-TRFIA was presented in Fig. 1. The assay of AFM1 was performed as follows: 50-μL standards or samples and 50 μL anti-AFM1 antibody in assay buffer were added to the well. After incubation with shaking at 25 or 37 °C for 1 h, the plate was washed four times with the washing buffer. Then, 100 μL of a diluted (1:100 *v*/*v*) Eu^{3+} goat anti-rabbit antibody in assay buffer was added into the wells and shaken gently for another 1 h. The plate was washed six times again and 100 μL of the enhancement solution was added. After incubating for 5 min, the fluorescence was measured using Auto $DELFIA_{1235}$ and the concentrations of AFM1 in the sample were determined from standard curves.

Table 2 Cross-reactivity of the anti-AFM1 antibody with aflatoxins

Aflatoxins	IC_{50} (μg/kg)	Cross-reactivity (%)
AFM1	0.173	100
AFB1	582	0.03
AFG1	>1000	<0.02
AFG2	>1000	<0.02

Results and Discussion

Optimization of AFM1–TRFIA

Incubation time and temperature can significantly influence the assay performance. Contrast test was carried out at 25 and 37 °C for AFM1 detection in full-cream sterilized milk when the concentration of the coating antigen (AFM1-BSA conjugate) and anti-AFM1 antibody were 0.25 and 1.0 mg/L, respectively. As shown in Fig. 2, when the reaction temperature was 37 °C, the response counts were higher than that at 25 °C. The lower the concentration of AFM1 was the more obviously different between 25 and 37 °C. Considering low concentration was more important to the detection of AFM1, 37 °C was selected as the incubation temperature in this study.

At 37 °C, different incubation times (15, 30, 45, 60 min) were compared for the first and second steps. As shown in Fig. 3, long incubation times could obviously enhanced the binding efficiency of the assay. However, when the incubation time exceeded 45 min, the fluorescence signal intensities of all standard points in both of the two steps reached a dynamic balance. Therefore, 45 min was selected as both the first incubation step and the second incubation step in this work.

The sensitivity of the indirect competitive AFM1-TRFIA depends on the concentrations of the AFM1-BSA conjugate and the anti-AFM1 antibody. In this study, AFM1-BSA conjugate was tested at concentrations ranging from 1 to 0.125 mg/L. Therefore, for determining the concentrations of AFM1 in milk, a set of standard curves were obtained by varying concentrations of AFM1-BSA conjugate and anti-AFM1 antibody. To evaluate the efficiency of the system, we select the values of IC10, IC50 and dynamic working range (IC20-IC80) as the parameters.

As shown in Table 1, the most sensitive system was obtained using combination 5, comparing with the other combinations. So, combination 5 is chosen as optimal in this work. The optimized standard curve for the indirect formats of the assay using the log-logit function is shown in Fig. 4, where $\mathrm{logit}(Y) = \ln[Y/(1-Y)]$, $Y = B/B_0$ and B_0 correspond to the fluorescence count at zero concentration.

For the optimized method, the limit of detection values (LOD, equal to IC10), IC50 and the dynamic working range (IC20–IC80) were 0.006 and 0.173 μg/kg and 0.022–1.334 μg/kg, respectively. And by now, other works using quantitative immunoassays were reported. A time-resolved fluorescent competitive immunochromatographic assay was developed for determination of AFM1 in raw milk with a dynamic range of 0.1–2.0 μg/kg (Tang et al. 2015), and not including the AFM1 limit in milk for EU legislation. The dynamic range of fluorescent microsphere immunochromatographic test strip assay of AFM1 was 0.01–0.32 μg/kg (Zhang et al. 2016). Similar to the above, a direct chemiluminescent enzyme immunoassay for

Table 3 Spiked curves of raw milk, skimmed milk, and semi-skimmed milk by indirect competitive AFM1–TRFIA

Types of milk	IC_{50} (μg/kg) (*n* = 3)	IC_{20}-IC_{80} (μg/kg) (*n* = 3)	Recovery[a] (intra-day precision, CV) (%)			
			Level (μg/kg)			
			0.05 (*n* = 3)	0.1 (*n* = 3)	0.5 (*n* = 3)	1.0 (*n* = 3)
Raw milk	0.188	0.027–1.292	98 ± 0.3 (5.4)	98 ± 0.3 (3.2)	89 ± 3.2 (7.2)	101 ± 6.2 (6.2)
Skimmed milk	0.171	0.021–1.322	101 ± 0.2 (4.1)	94 ± 0.2 (2.4)	103 ± 2.1 (4.1)	94 ± 2.1 (2.2)
Semi-skimmed	0.197	0.029–1.332	91 ± 0.1 (2.2)	99 ± 0.4 (4.0)	95 ± 1.5 (3.2)	89 ± 6.1 (6.8)

CV coefficient of variation

[a] The report data are the mean ± SD

determination of AFM1 in milk was reported with a dynamic range of 0.002–0.0075 μg/kg (Vdovenko et al. 2014). Peng et al. (2016) achieved the quantification of AFM1 in milk with a detection range of 0.005–0.405 μg/kg by indirect competitive enzyme-linked immunosorbent assay. However, all of these methods were excluded the maximum legal levels in China and were not suitable for commercial use of AFM1 determination. Compared to the above, the dynamic working range in this study is more suitable and sufficient for the determination of AFM1.

Cross-Reactivity and Limit of Quantification for the AFM1–TRFIA

Based on the standard curve (Fig. 4), the extent of cross-reactivity (CR) and limit of quantification (LOQ) were assessed. As a parameter to evaluate specificity, CR was assessed by determining the IC_{50} values in the indirect competitive AFM1-TRFIA. AFB1, AFG1, and AFG2 spiked in full-cream sterilized milk were selected to test for CR. The CR values were calculated using the following formula: CR = IC_{50} of AFM1/(IC_{50} of competitor) × 100%. As shown in Table 2, no CR was observed with other aflatoxins, such as AFB1, AFG1, and AFG2. The LOQ value was obtained by the analysis of blank full-cream sterilized milk samples. LOQ is the concentration corresponding to the mean concentration of 20 blank samples plus ten times of standard deviation (SD). Using the optimized method, the LOQ value was 0.022 μg/kg well below the maximum legal levels in EU and China.

Matrix Interference

To evaluate and correct for the matrix interference caused by sample matrices, AFM1 concentration in spiked milk samples was measured. First, compared to the full-cream milk, the different types of milk were evaluated, including raw milk, semi-skimmed, and skimmed milk. The obtained samples were measured by the optimized system and the results were shown in Table 3.

The value of IC50 and the dynamic working range were used as parameters to compare the matrix interference caused by different types of milk, in which no obvious difference was observed, combining with Table 1. Recoveries were almost the same for different types of milk in the optimized system. So, the method was applicable for the raw milk, full-cream, semi-skimmed, and skimmed milk. Then, the recovery and CVs of diluted full-cream sterilized milk samples were measured, in which the milk was mixed with PBS at 1:1, 1:3, 1:7, and 1:15 in volume, respectively. And the concentrations of the diluted milk samples were 0.05, 0.10, and 0.25 μg/kg. Results were shown in Table 4.

Table 4 Recovery and CVs of AFM1 from spiked full-cream sterilized milk samples

Spiked AFM1 (μg/kg)	Undiluted milk sample[a]		Dilutions of milk sample[a]							
	(*n* = 3)		1/2 (*n* = 3)		1/4 (*n* = 3)		1/8 (*n* = 3)		1/16 (*n* = 3)	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
0.05	89.5 ± 5.2	5.8	87.6 ± 7.2	8.2	98.1 ± 5.0	5.1	95.9 ± 0.6	0.6	86.9 ± 0.9	1.0
0.10	88.1 ± 4.4	5.0	91.7 ± 3.2	3.5	96.3 ± 2.6	2.7	99.2 ± 1.0	1.0	106.2 ± 1.3	1.2
0.25	92.2 ± 2.7	2.9	89.7 ± 1.1	1.2	90.1 ± 1.4	1.6	103.3 ± 2.8	2.7	102.0 ± 1.7	1.7

[a] The report data are the mean ± SD

Table 5 Analysis of four spiked full-cream sterilized milk samples undiluted of AFM1 by indirect competitive AFM1-TRFIA

Group no.	Spiked milk (μg/kg)	Within assay[b] (n = 4)			Between assays[c] (n = 4)	
		Average of the found[a] (μg/kg)	Recovery (%)	CV (%)	Recovery[a] (%)	CV (%)
1	0.05	0.048 ± 0.003	95.0–98.5	3.8–8.2	96.25 ± 1.5	1.6
	0.1	0.097 ± 0.006	93.3–100.8	1.5–8.4	97.31 ± 3.3	3.4
	0.25	0.254 ± 0.008	99.1–102.5	2.5–4.2	101.45 ± 1.6	1.6
	0.5	0.518 ± 0.038	96.0–109.0	5.7–8.6	103.5 ± 5.8	5.6
2	0.05	0.049 ± 0.003	94.5–100.5	3.8–7.0	97.63 ± 2.5	2.6
	0.1	0.099 ± 0.005	90.8–103.5	4.1–7.5	98.50 ± 5.5	5.5
	0.25	0.245 ± 0.008	94.4–99.7	0.9–4.2	98.08 ± 2.5	2.5
	0.5	0.543 ± 0.030	102.5–116.0	1.8–8.8	108.63 ± 5.8	5.3
3	0.05	0.047 ± 0.003	89.0–101.5	4.1–5.8	93.38 ± 5.6	6.0
	0.1	0.096 ± 0.005	95.3–98.5	2.2–6.5	96.38 ± 1.5	1.6
	0.25	0.249 ± 0.009	97.6–102.8	2.5–3.9	99.75 ± 2.2	2.2
	0.5	0.496 ± 0.030	93.0–109.0	2.3–7.8	99.25 ± 7.1	7.2
4[d]	0.05	0.051 ± 0.003	94.0–106.0	4.0–6.7	101.38 ± 5.1	5.1
	0.1	0.097 ± 0.004	92.3–103.3	2.7–6.2	97.19 ± 4.5	4.7
	0.25	0.251 ± 0.007	94.2–104.3	1.9–5.1	100.28 ± 4.7	4.6
	0.5	0.523 ± 0.032	98.0–112.5	5.0–8.1	104.63 ± 7.2	6.9
5[e]	0.05	0.053 ± 0.004	101.5–112.0	3.4–9.2	106.63 ± 4.6	4.3
	0.1	0.093 ± 0.005	88.0–97.0	4.0–7.9	92.69 ± 4.3	4.6
	0.25	0.253 ± 0.006	97.2–105.8	1.8–4.1	101.23 ± 3.6	3.5
	0.5	0.483 ± 0.029	92.0–99.0	5.1–8.5	96.63 ± 3.2	3.3

[a] The report data are the mean ± SD

[b] The assays are carried out in four replicates on the same day

[c] The assays are carried out in four different days

[d, e] The assays are carried out in different laboratory by different operators

The obtained results showed that there was no influence on the results of AFM1 detection, whether the matrix had been diluted. The recovery and CVs were in range of 86.9–106.2% and 0.6–8.2% that were no significant differences among the various dilution rates of the milk samples. Therefore, the matrix interference of milk appears to be negligible.

Recovery and Precision of the AFM1-TRFIA

Four spiked full-cream sterilized milk samples that undiluted (Table 5) with different concentrations varying from 0.05 to 0.5 μg/kg were analyzed. It showed that the recovery values was in the range of 88.0–116.0% with CVs (n = 4) less than

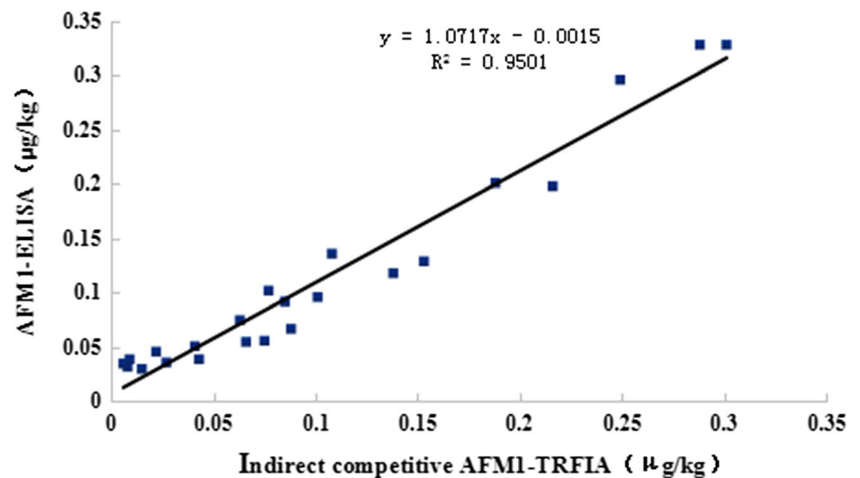

Fig. 5 Correlation between indirect competitive AFM1-TRFIA and AFM1-ELISA for the determination of AFM1 in undiluted full-cream sterilized milk samples

9.2%. Also, the values of recovery between assays obtained by the use of the indirect competitive TRFIA method day by day ($n = 4$) were in the range of 92.69–108.63% and CVs less than 7.2%. Repeatability (intra-laboratory variability) and reproducibility (inter-laboratory variability) of the observed results were assessed. The within-laboratory percent CVs that calculated among replicates obtained in the same laboratory were generally low and ranged from 1.2 to 4.5%. Values of reproducibility among results in different laboratories were in the range of 0.8–5.0%. Both of the repeatability value and reproducibility value did not exceed 5.0%. The results demonstrated that the method had a good repeatability and reproducibility. Meanwhile, the results from the test above revealed that the indirect competitive AFM1-TRFIA kit was stable for 4 months at least.

Comparison with the AFM1-ELISA

To embody the benefits of the method, 23 full-cream sterilized milk samples undiluted from different marks that purchased in supermarket were analyzed by the indirect competitive TRFIA and AFM1-ELISA, respectively. Results were shown in Fig. 5 The concentration of AFM1 was below the detection limit of AFM1-ELISA (0.075 μg/kg) in ten milk samples. Data obtained from the milk samples by TRFIA and ELISA were in good agreement. Paired *t* test used for statistical analysis was applied to the data. Results there showed that the difference between two methods was not statistically significant ($P > 0.05$). For all the samples, the concentration measured was in the range of 0.006–0.301 μg/kg, lower than the maximum legal levels in China. And among these, the maximum value concentration of five full-cream sterilized milk samples imported from the EU was 0.043 μg/kg. This finding also suggested that the milk sold in Wuxi were generally safe for customers.

Conclusions

To protect the human health, low level of AFM1 was expected in milk. The sensitivity of the methods for determination of AFM1 should as high as possible. The sensitivity (LOD 0.006 μg/kg, LOQ 0.022 μg/kg) is high in this paper, and the dynamic working range (0.022–1.334 μg/kg) is suitable for the AFM1 determination in milk and is more sufficient than the values reported in other works by now using quantitative immunoassays.

In this study, we found that the interference of matrix could be negligible. The method was applicable for the full-cream, semi-skimmed, skimmed, and raw milk as well. And no cross-reactivity was observed with other aflatoxins. With good repeatability and stability, the indirect competitive AFM1-TRFIA is a sensitive and adequate tool for the identification of AFM1 contamination status in milk. For further practical use, we will apply this method to rapid determination (Guo et al. 2015). This system will also be extended to determination of more milk products, such as cheese, ice cream, and milk power. The detailed work of the study will be reported in the future.

Compliance with Ethical Standards

Funding None.

Conflict of Interest Mingming Guo declares that she has no conflict of interest. Bin Zhou declares that he has no conflict of interest. Zijian Huang declares that he has no conflict of interest. Chuncheng Zhao declares that he has no conflict of interest. Jue Zhang declares that she has no conflict of interest. Biao Huang declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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