Establishment of a Rapid and Sensitive Chemiluminescence Enzyme Immunoasay for Aflatoxin M_1: Verified by HPLC

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Abstract: A rapid and sensitive chemiluminescence enzyme immunoassay method (CLEIA) was established to detect Aflatoxin M_1 (AFM_1) in milk, which was Verified by high performance liquid chromatography (HPLC). It only takes 30 minutes. Optimized conditions included antibody dilution ratio and enzyme conjugate, ionic strength, pH value and organic solvent. Results: The 50% inhibitory concentration(IC_{50}) and the detection limit of the CLEIA were 0.08ng/mL and 0.024ng/mL, the recovery ranged from 86.94% to 114.49% in dairy products. The correlation was 99.7% between this method and HPLC .

1 INTRODUCTION

AFM_1 is produced by hydroxylation when mammals ingesting crops contaminated with Aflatoxin B1. It has a destructive effect on liver tissue, with Strong carcinogenicity and mutagenicity. At present, a lack of effective method for prevention and detoxification, so the monitoring of AFM_1 is an important means to prevent and control.

At present, the analytical method mainly include: high performance liquid chromatography (Shuib et al., 2017) time-resolved fluorescence (Gao et al., 2017), quantum dot immunoassay (Bailey et al., 2004) and enzyme-linked immunosorbent assay (Radoi et al., 2008) etc. Instrument method are expensive, laborious, time-consuming, and sample pretreatment cumbersome. In recent years, the limited standard of AFM_1 is decreasing (eg: Commission regulation EU No 165, 2010; GB 2761-2017 limits on mycotoxins in food, 2017), so it is necessary to establish simple, rapid and high sensitivity detection method to quantify and confirm the AFM_1 in dairy products. However, chemiluminescence enzyme immunoassay is combination of ELISA and chemiluminescence, the detection sensitivity is 10 ~ 102 orders of magnitude than conventional ELISA (Zhao et al., 2009).

2 MATERI ALS AND METHODS

Instruments: Luminoskan Ascent and its software (Thermo, USA), 96-well white polystyrene plates (Costar), AFM_1 immunoaffinity column (Own laboratory), High performance liquid chromatography.

Reagents: AFM_1 standard solutionantigen and anti-AFM_1 monoclonal antibody were got from our own laboratory. IgG-HRP was purchased from Sigma, Luminol chemiluminescent substrate was purchased from Helisence (Shanghai, China)

2.1 CLEIA Operation

Chemiluminescent plate were coated with 120μL of AFM_1 antigen per well for the night at 4℃ washed 4 times with PBS, added 320μL5% skim milk per well, which incubated for 2h at 37℃, after washing 4 times, added 50μLstandard solution or sample solution, 50μL antibody diluotin and 100μL IgG-HRP per well at 37°C for 30min then washed 4 times, at last added 100μL Luminol chemiluminescent substrate per well and get the relative light unit (RLU).

2.2 Sample Preparation

Weighing 1g milk powder in centrifugal tube, adding standard, adding 5ml acetonitrile (liquid milk take 3ml, add 3ml acetonitrile), vortex 5min and

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centrifugal 15 minute for 4500r/min, remove the underlying liquid and dry it with a nitrogen blower , then dissolve the residue with 5% skimmed milk for test.

Sample pretreatment for liquid chromatography is referred to 2016 National Food Safety Standard determination of Aflatoxin M in Food (GB, 2016) .

3 RESULTS AND DISCUSSION

3.1 Optimization of the AFM_1 CLEIA Reaction System

The concentration of the coating antigen was optimized by the checkerboard titration, the coated concentration between 0.25μg/mL to 2μg/mL. The results show that 0.5μg/ml is the best coated concentration. When antibody concentration was too low, it led to a small RLUmax. Therefore, antibody concentrations were selected from 1:2000 to 1:8000. Finally, the best antibody dilution is 1: 2000.

The RLUmax/IC50 ratio was used as a parameter to judge the impact of factors. Research the effects of enzyme dilution , ionic strength, pH value and methyl alcohol on CLEIA. The results demonstrate that optimum conditions when enzyme concentration at 1: 500, ionic strength was 5mM , methyl alcohol was 0 and pH was 7.0. As show in Figure 1-4. This indicates that neutral buffer is beneficial to the combination of AFM_1 antigen- antibody.

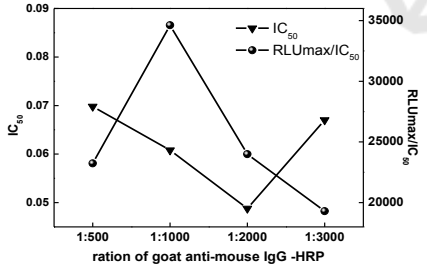

Figure 1: Effects of enzyme dilution on CLEIA.

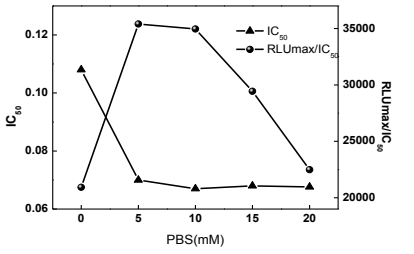

Figure 2: Effects of ionic strength on CLEIA.

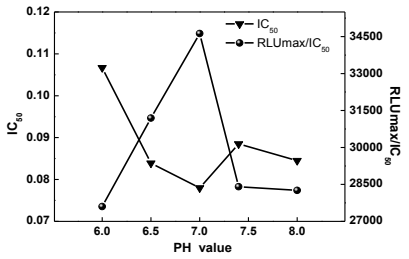

Figure 3: Effects of pH on CLEIA.

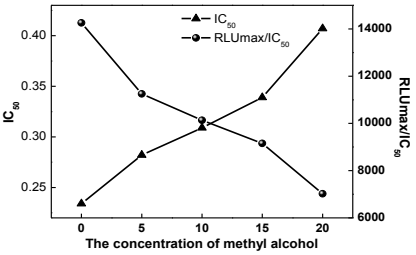

Figure 4: Effects of methyl alcohol on CLEIA.

3.2 Establishment of CLEIA Standard Curve

Based on the optimization results, the standard curve of AFM_1 immunoassay was established in Figure 5 . The linear equation was Y = -55.228 +154.73 (R2 = 0.9916), The IC50 was 0.08ng/ml, the detection limit was 0.024ng/ml.

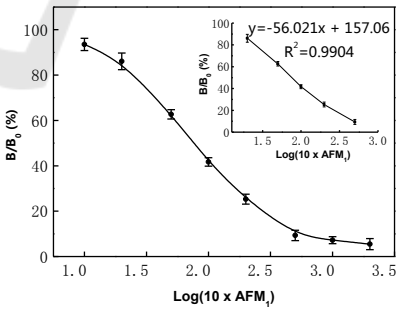

Figure 5: Competitive inhibition curve for AFM_1 by CLEIA.

3.3 Verification by High Performance Liquid Chromatography

A series of AFM_1 standard solutions (15, 10, 5, 2, 1, 0.5 and 0.1ng/mL) were prepared with 10%

acetonitrile aqueous solution, chromatographic conditions reference to GB of Aflatoxin M1. The characteristic absorption peak of AFM_1 was obtained and retention time was 8.993min in the set chromatographic conditions. The SNR was 3:1 as the minimumdetection limit, which was 0.075ng/mL and the quantitative limit was 0.3ng/mL. As show in Figure 6.

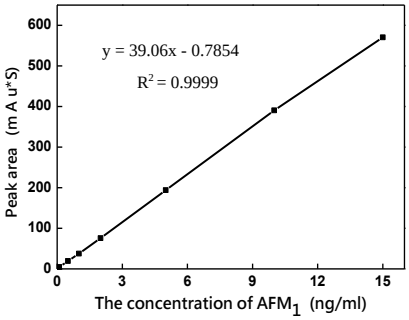

Figure 6: HPLC stand curve for AFM_1.

3.3.1 Recovery Test of Spiked Samples

To verify the accuracy and reliability of CLEIA, the recovery experiments were carried out in different dairy products, moreover it made a correlation with HPLC. As shown in Table 1, the recovery ratio of CLEIA was 86.94%-114.49, the coefficient of variation was 0.81%-7.26%; the recovery by HPLC was 85.25%-98.62%, and the coefficient of variation was 0.72%-9.64%. Both methods have good accuracy and Precision.

Table 1: Recoveries of AFM_1 in diferrent dairy products (n=4).

Sample number	CLEIA		HPLC		
	Spiked value (ng/ml)	Recovery ratio (%)	CV (%)	Recovery ratio (%)	CV (%)
1	0.1	90.78	5.15	92.76	9.64
	0.5	109.08	0.81	98.62	0.72
2	0.1	114.49	7.26	96.14	2.95
	0.5	86.94	3.74	85.25	3.37

3.3.2 Determination of AFM_1 in Naturally Sample

Figure 7 shows in adding standard solution of AFM_1 (0.1ng/ml, 0.5ng/ml, 1ng/ml) in a milk sample (The milk sample used does not contain AFM_1). Using CLEIA and HPLC to test. The data show a high degree of correlation between them (R2 = 0.997).

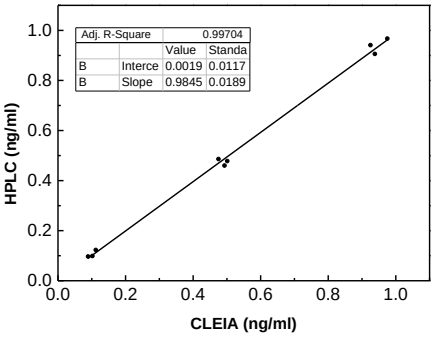

Figure 7: Correlation between the CLEIA and HPLC.

From Table 2, it can conclude that the content of AFM_1 in 10 samples detected by CLEIA and HPLC was lower than the national limit. Besides AFM_1 was not detected in ELISA in 10 samples, indicating that the content of AFM_1 was lower than the minimum detection line of this method, which is consistent with the result of HPLC detection.

Table 2: AFM_1 detection in naturally sample.

Sample number	Detected value（ng/mL）	
	CLEIA	HPLC
1	ND	0.0089
2	ND	ND
3	ND	ND
4	ND	ND
5	ND	ND
6	ND	ND
7	ND	ND
8	ND	0.0137
9	ND	ND
10	ND	ND
Note: “ ND ”（Not detected）		

4 CONCLUSIONS

In the study, CLEIA reaction system has been comprehensively optimized. Finally, the immunoassay method of AFM_1 was established, and the sensitivity was 0.024ng/ml. The recoveries ranged from 86.94% to 114.49%, Meanwhile correlation between the detection results of CLEIA and high performance liquid chromatography was 99.7%, indicating that established method can be applied to rapid determination of AFM_1 in milk.

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