Validation of an Improved ELISA Method for Detection of Aflatoxin M1 in Milk and Dairy Products

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INTRODUCTION

Aflatoxins are toxic, carcinogenic metabolites produced by a variety of molds, such as *Aspergillus flavus* and *Aspergillus parasiticus*, that can develop in grains, nuts, cottonseed and other commodities associated with human food or animal feeds. Aflatoxins may cause decreased animal production and are found as four subtypes, B1, B2, G1 and G2, with aflatoxin B1, the most prevalent, being the most potent known carcinogen (**1**) and a likely etiologic agent of human liver cancer (**2**). When dairy animals are fed contaminated feed, aflatoxin B1 is hydroxylated in the liver, yielding Aflatoxin M1 (AFM1), which is then secreted in the milk. AFM1 remains toxic and stable in processed milk and dairy products for human consumption.

The regulated limits for the presence of AFM1 vary by region and the target consumer. The European Union (EU) has established acceptable limits for AFM1 concentrations in milk of 50 ng/kg and 25 ng/kg for infant milk products (**3**).

For routine quality control in dairy laboratories, the enzyme-linked immunosorbent assay (ELISA) offers a practical approach to quantitative screening. An ELISA method for AFM1 detection should deliver the required precision, accuracy, and quantitation capability across diverse dairy matrices, while providing rapid results through a simple workflow.

PURPOSE:

The purpose of this work was to validate a new ELISA design, **Aflatoxin M1 ELISA Rapid**, to determine its suitability as an efficient method for quantitative detection of AFM1 in milk and common dairy products.

Assay Workflow

1) Incubate the samples in the plate for 30 minutes.
2) Wash the plate.
3) Incubate with the AFM1–HRP conjugate for 15 minutes.
4) Wash the plate again.
5) Incubate with the HRP substrate for 15 minutes.
6) Stop reaction and read the plate.

Analytical Range and Sample Compatibility

- **Analytical range:** 5–100 pg/mL.
- **High-concentration samples:** The included Assay Diluent can be used to dilute samples with AFM1 concentrations above the analytical range.
- **Compatible matrices:** Milk, yogurt (*), and cheese (**).

* Yogurt samples are diluted 1:4 in Assay Diluent before step 1 of the procedure.
** Cheese samples are extracted 1:5 in absolute methanol, dried and reconstituted in Assay Diluent before step 1 of the procedure.

REFERENCES:

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2. Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *Am J Clin Nutr.* 2004 Nov;80(5):1106-22. doi: 10.1093/ajcn/80.5.1106. PMID: 15531656.
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REGISTERED TRADEMARKS

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METHOD PRINCIPLE:

Hygiena's **Aflatoxin M1 ELISA Rapid** is a competitive, enzyme-linked immunosorbent assay. In this method, polystyrene microwells coated with an AFM1-specific antibody are first incubated with the samples and AFM1 standards. After a washing step, an AFM1-horseradish peroxidase (HRP) conjugate is added and binds to the free AFM1 binding sites in the wells. After a new wash, incubation with an HRP substrate generates a colored product that is inversely proportional to the amount of AFM1 in the standard or sample. Finally, the optical density (OD) values obtained from the AFM1 standards are used to prepare a dose-response curve, and AFM1 concentration values in the samples are interpolated.

Calibration curve

To prepare a dose-response curve, the OD_{450nm} values from the non-zero AFM1 standards (5, 10, 25, 50 and 100 pg/mL) are expressed as a % of that of the zero standard (**Table 1**). The resulting %B/B0 values are plotted against the corresponding AFM1 concentration values to obtain a sigmoidal calibration curve that fits a 4-parameter logistic (4PL) regression model (**Figure 1**).

Table 1. %B/B0 ratios obtained from AFM1 standards, in three independent assays. For each assay, B and B_0 values correspond to mean OD_{450nm} values from three well replicates.

		%B/B0	%B/B0	%B/B0	Mean (%B/B0)	%CV
AFM1 Standards (pg/mL)	100	29,7%	29,8%	27,6%	29,0%	4,2%
	50	42,7%	43,1%	42,4%	42,8%	0,8%
	25	65,5%	64,7%	65,3%	65,2%	0,7%
	10	84,7%	81,6%	82,3%	82,9%	2,0%
	5	90,1%	90,2%	88,9%	89,7%	0,8%
	0	100,0%	100,0%	100,0%	100,0%	0,0%

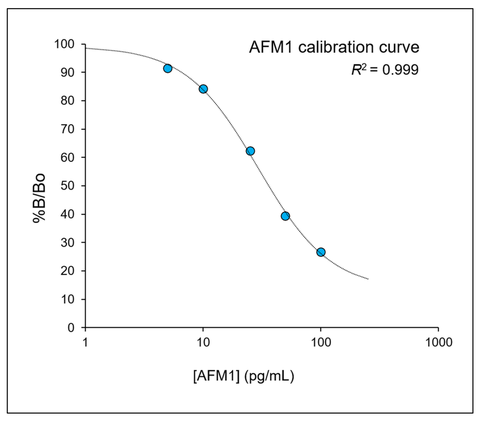


Figure 1. Representative Standard Curve

ASSAY CHARACTERISTICS:

Sensitivity and selectivity

The Limit of Detection (LoD) of the assay (3.7 pg/mL) was estimated as the theoretical AFM1 concentration, interpolated from the calibration curve, using the average OD_{450nm} - 2xSD obtained with 18 determinations of the 0 pg/mL standard. Cross-reactivity with non-M1 aflatoxins was tested in samples containing 2 x 10^5 pg/mL of aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) or G2 (AFG2). The result of the assay was negative in all cases (**Table 2**).

Linearity in Assay Diluent

A whole milk blank sample was spiked with 400 ppt AFM1 and further diluted in Assay Diluent to obtain samples with AFM1 concentrations (5, 10, 25, 50 and 100 pg/mL) within the calibration range. Each sample was tested in replicates (n=8), and the means of the test results were plotted against the spiked AFM1 concentration values, showing linearity (**Figure 2**).

Table 2. Sensitivity and selectivity of the assay.

Aflatoxin	Concentration (pg/mL)	Assay result
AFM1	3.7 (= LoD)	positive
AFB1	2x10^5	< LoD
AFB2	2x10^5	< LoD
AFG1	2x10^5	< LoD
AFG2	2x10^5	< LoD

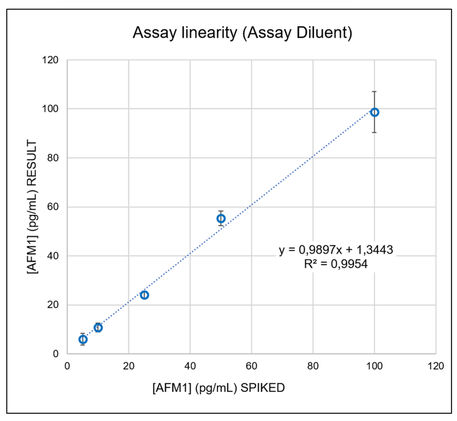


Figure 2. Assay Linearity with Milk Samples Diluted in Assay Diluent

ASSAY PERFORMANCE:

Matrix study: Raw milk

A blank sample of raw milk was spiked with 5, 10, 16, 25, 32, 50, 75 and 100 pg/mL AFM1. Each sample was assayed in triplicate (n=3), and the individual OD_{450nm} values were used to interpolate test results (3 results for each sample). The results showed ~90% average recovery in the 5 pg/mL – 100 pg/mL AFM1 concentration range, with a maximum %CV of 16,2% (**Table 3**), and linearity when plotted against the spiked AFM1 concentrations (**Figure 3**).

Table 3. Average recovery values obtained from raw milk samples spiked with AFM1. For each contamination level, the coefficient of variation (CV) was calculated as the ratio between the standard deviation and the average AFM1 result.

		Mean assay result (pg/mL)	Mean recovery	%CV
AFM1 in raw milk (pg/mL)	100	97,7	98%	5,7%
	75	70,9	95%	7,7%
	50	47,4	95%	3,2%
	32	28,6	89%	0,6%
	25	23,2	93%	4,4%
	16	15,3	95%	2,8%
	10	8,1	81%	5,1%
	5	4,3	87%	16,2%

Matrix study: Skimmed milk

A blank sample of skimmed milk was spiked with 5, 8, 10, 16, 25, 32, 50, 75 and 100 pg/mL AFM1. Each sample was assayed in replicates (n=8), and the mean OD_{450nm} values from adjacent wells were used to interpolate the test results (4 values per sample). The results showed ~90% average recovery in the 8 pg/mL – 100 pg/mL AFM1 concentration range, with a maximum %CV of 10,2% (**Table 4**), and linearity when plotted against the spiked AFM1 concentrations (**Figure 4**).

Table 4. Average recovery values obtained from skimmed milk samples spiked with AFM1. For each contamination level, the coefficient of variation (CV) was calculated as the ratio between the standard deviation and the average AFM1 result.

		Mean assay result (pg/mL)	Mean recovery	%CV
AFM1 in skimmed milk (pg/mL)	100	80.0	80%	4,2%
	75	64.6	86%	3,3%
	50	43.4	87%	2,2%
	32	27.9	87%	5,0%
	25	22.4	90%	2,0%
	16	15.8	99%	8,0%
	10	9.9	99%	10,2%
	8	8.4	106%	7,9%
	5	7.2	144%	12,6%

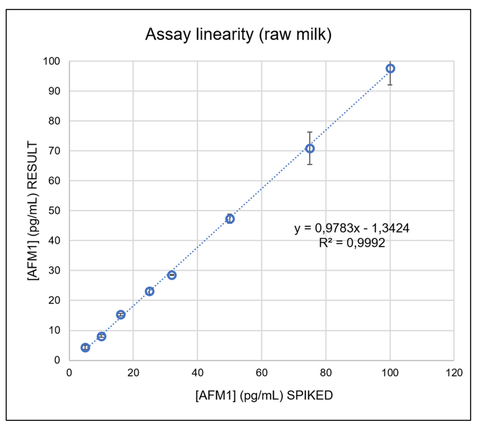


Figure 3. Assay Linearity in Raw Milk

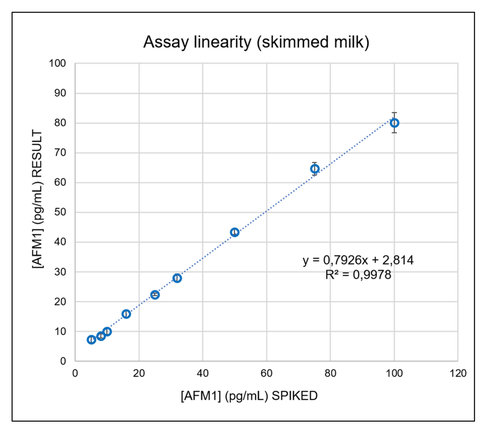


Figure 4. Assay Linearity in Skimmed Milk.

Matrix study: Representative dairy matrices

Blank samples of raw milk, homogenized whole milk, skimmed milk, reconstituted skimmed milk powder, cheese and yogurt were spiked with 50 or 100 pg/mL AFM1. For each matrix, two or three independently spiked samples were prepared and assayed in replicates (n=8), and the individual OD_{450nm} values were used to interpolate test results (8 results for each sample). Mean recovery was 100% for raw milk and yogurt, 94% for whole milk, and 90% for skim milk. Mean recovery from cheese was 75%. (**Table 5**).

Table 5. Average recovery values obtained from samples of representative dairy matrices spiked with AFM1. The coefficient of variation (CV) was calculated as the ratio between the standard deviation and the average AFM1 result.

Matrix	AFM1 (pg/mL)	Mean assay result (pg/mL)	Mean recovery	%CV
Raw milk	50	49,6	99%	2,5%
	50	50,0	100%	5,3%
	50	51,2	102%	3,1%
Homogenized whole milk	50	47,5	95%	6,6%
	50	46,0	92%	2,6%
	50	47,0	94%	5,4%
Skimmed milk	50	45,5	91%	6,0%
	50	45,9	92%	5,3%
	50	44,1	88%	4,1%
Skimmed milk powder	50	36,5	73%	3,7%
	50	36,9	74%	5,7%
	50	36,5	73%	5,7%
Cheese	100	70,2	70%	8,9%
	100	80,4	80%	5,3%
Yogurt	50	49,1	98%	6,4%
	50	50,1	100%	4,5%
	50	50,3	101%	4,1%

SUMMARY:

Hygiena's **Aflatoxin M1 ELISA Rapid** is a fit-for-purpose solution for detecting AFM1 in milk and common dairy products. Interested in participating in beta testing? Please contact us at marevalo@hygiena.com.