NEOGEN® CORPORATION

Lansing, Michigan USA

A Sensitive ELISA for the Detection and Quantitation of Aflatoxin M_1 (AFM_1) in Milk and Dairy Products

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ABSTRACT

Aflatoxin is widely considered to be one of the most potent naturally-occurring carcinogens. Aflatoxin M_1 is a toxic metabolite that is excreted in milk by livestock that have consumed feed contaminated with aflatoxin B_1. Aflatoxin M_1 in milk and dairy products is considered a risk to human health and as a result there is an established European Union (EU) limit of 0.05 µg/L or 50 part per trillion (50 ppt) in milk and reconstituted milk powder. Veratox for Aflatoxin M_1 is a competitive immunoassay (ELISA) in microwell format for the detection and quantitation of aflatoxin M_1 (AFM_1) in milk and dairy products. The assay is very sensitive with a limit of detection of 5 ppt and a quantitative range from 5–100 ppt. The assay is very specific to AFM_1 and has no cross reactivity with any other aflatoxins including AFM_2. The assay is performed by adding aflatoxin standard controls and samples to aflatoxin M_1 monoclonal antibody-coated wells and, during a 20 minute incubation the AFM_1 will bind to the antibody. After the first washing step, aflatoxin M_1 that has been conjugated to horseradish peroxidase (AFM_1-HRP) is added and incubated for 10 minutes in which the conjugate will bind to any remaining unbound antibody. After the second washing step, substrate is added, which reacts with the bound conjugate to produce blue color. The color development is inversely proportional to the aflatoxin M_1 in the sample; more blue color means less aflatoxin M_1. The test is read in a microwell reader to yield optical densities. The optical densities of the controls form the standard curve, and the sample optical densities are plotted against the curve to calculate the exact concentration of aflatoxin M_1. ELISA results correlated very well with results of reference HPLC method of naturally contaminated whole milk powder samples with low, medium and high levels of AFM_1.

INTRODUCTION

Aflatoxin M_1 is a toxic metabolite of aflatoxin B_1 which is produced by molds such as *Aspergillus falvos* and *Aspergillus parasiticus*.

Aflatoxins are carcinogenic and can be present in grains and animal feeds.

When animals are fed grains and feeds contaminated with aflatoxins, aflatoxin B_1 is converted by hydroxylation to aflatoxin M_1, which is subsequently secreted in the milk of lactating cows. Many governments have established regulations for the maximum permissible amounts of aflatoxin M_1 in different dairy products. In the EU, the limit for AFM1 in milk and reconstituted milk powder is at 0.005 ng/mL (0.05 µg/L or 50 ppt. In the USA, the Food and Drug Administration (FDA) established an action level of 0.5 ng/mL (0.5 µg/L or 500 ppt) in milk.

SAMPLE PREPARATION AND EXTRACTION

LIQUID MILK

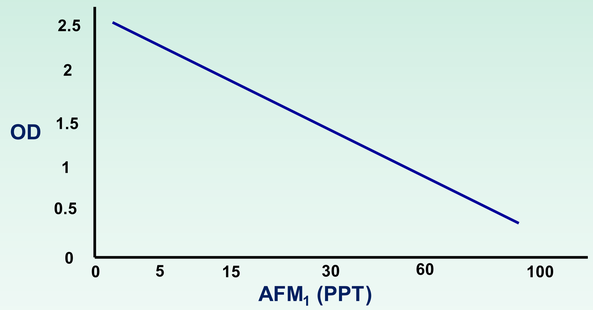
1. Centrifuge milk samples to remove fat for 10 minutes at 3500 g at 10°C (50°F).
2. After centrifugation, carefully separate and discard the upper cream layer from the defatted supernatant.
3. The supernatant (skimmed milk) will be used directly in the test (100 µL per well)

DRIED MILK POWDER

1. Weigh 10 g of milk powder in a flask. Add 100 mL of distilled water.
2. Stir to dissolve and homogenize.
3. Extract in a rotary shaker for 30 minutes.

NOTE: If whole milk powder is used, continue with steps 4 and 5. If not testing whole milk powder, proceed to step 6.

4. Centrifuge milk samples to remove fat for 10 minutes at 3500 g at 10°C (50°F).
5. After centrifugation, carefully separate the upper cream layer from the defatted supernatant. The cream layer can be discarded.
6. The supernatant (skimmed milk) will be used directly in the test (100 µL per well).



TEST PROCEDURE

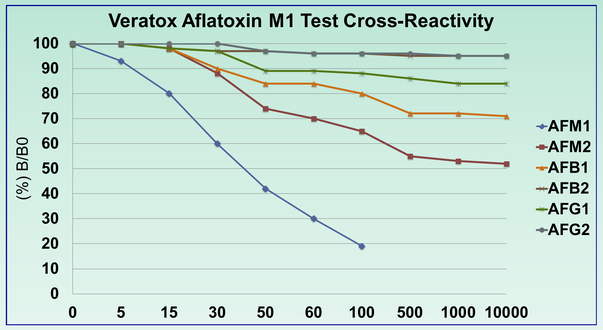
1. Add 100 µL per well of control or samples in duplicate wells and incubate for 20 minutes with continous shake.
2. Wash 5 times with PBS-Tween.
3. Add 100 µL per well of aflatoxin conjugate and incubate for 10 minutes with continous shake.
4. Wash 5 times with PBS-Tween.
5. Add 100 µL per well of ready-to-use substrate and incubate for 15 minutes with a continous shake.
6. Add 100 µL per well of stopping reagent.
7. Read using ELISA reader with a 650 nm filter.
8. Calculate concentration of AFM1 expressed as ppt.

TEST CROSS-REACTIVITY

The test is very specific for AFM_1. There is no cross-reactivity with AFM_2 or other aflatoxins (B_1, B_2, G_1 and G_2).

AFLATOXIN M_1 TEST CROSS-REACTIVITY

Compounds	50% inhibition (pg/mL)	Cross-reactivity (%)
AFM_1	42	100
AFB_1	>10,000	<0.40
AFB_2	>10,000	<0.40
AFG_1	>10,000	<0.40
AFG_2	>10,000	<0.40



MILK AND MILK POWDER SPIKING RECOVERY

RECOVERY OF SPIKED LIQUID SKIM MILK

AFM_1 spiked level (PPT)									
0		5		10		20		50	
AFM_1 recovered (PPT)									
PPT	%	PPT	%	PPT	%	PPT	%	PPT	%
5	-	9	90	12	80	21	84	57	104
Mean recovery (%)									
90									

RECOVERY OF SPIKED DRIED SKIM MILK

AFM_1 spiked level (PPT)									
0		50		100		200		500	
AFM_1 recovered (PPT)									
PPT	%	PPT	%	PPT	%	PPT	%	PPT	%
31	-	67	83	99	76	176	76	459	86
Mean recovery (%)									
80									

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CHEESE AND BUTTER SPIKING RECOVERY

Different European cheese varieties and USA-made butter were spiked with AFM_1 at 100 and 250 pg/g sample (20 and 50 pg/mL of final solution). The dichloromethane (DCM) method was used to extract aflatoxin M_1 from samples. After evaporation, the residue was reconstituted in 10% NFDM solution.

TWO OPERATORS AND THREE DIFFERENT KIT LOTS (N=6)

Product	AFM_1 spiked levels (PPT)				
	0	100		250	
	Recovery				
	ppb	ppb	%	ppb	%
Butter	18	88	75	216	81
Vintage cheddar cheese	29	96	74	219	79
Triple cream brie cheese	19	82	69	214	92
Parmigiano reggiano cheese	36	93	68	214	75
Mean recovery	77%				

RECOVERY OF CERTIFIED REFERENCE MATERIAL (CRM):

Whole powder milk certified reference material (CRM) of low, medium and high levels of aflatoxin M_1 were analyzed by HPLC and obtained from European Commission; DG-JRC-IRMM, Geel, Belgium were analyzed by Veratox for Aflatoxin for M_1 test. Data, as shown below, correlated very well with that of the reported reference materials.

RECOVERY OF CRM SAMPLES OF WHOLE POWDER MILK

HPLC Values (ppt ±SD)					
< 20 ppt		111 ± 18		440 ± 60	
Veratox AFM_1 test recovery (n=18)					
ppt ± SD	%	ppt ± SD	%	ppt ± SD	%
34 ± 12	–	102 ± 12	92	423 ± 26	96

VERATOX FOR AFLATOXIN M_1

- Veratox for Aflatoxin M_1 (AFM_1) can be used to detect and quantify AFM_1 in liquid milk, powdered milk, butter, cheese and other dairy products
- Monoclonal antibody-based competitive ELISA
- 45 minute assay (20:10:15 min.) – shortest in the market for this level of detection
- Limit of detection: 4.3 ppt (4.3 pg/mL or 0.0043 ng/mL)
- Limit of determination: 5 ppt (5 pg/mL or 0.005 ng/mL)
- Standard controls: 0, 5, 15, 30, 60 and 100 ppt of AFM_1
- Range of determination: 5–100 ppt
- Inter-assay variability: 4.6%
- Intra-assay variability: 3.4%
- Cross-reactivity: No cross-reactivity with AFM_2 or other aflatoxins (B_1, B_2, G_1 and G_2).
- Recovery:
  - Liquid milk: 90%
  - Skim milk powder: 94%
  - CRM whole milk powder: 94%
  - Cheeses and butter: 77%