Sensitive and Fast Analysis of Aflatoxin M1 in Milk at Picogram Levels using LC-MS-MS Analysis and Sample Preparation using Interference Removal Solid Phase Extraction

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Introduction

Aflatoxins are secondary metabolites produced by fungi of the *Aspergillus* genus(1). They are potent natural hepatocarcinogens and have been classified by the International Agency for Research on Cancer (IARC) as group 1 carcinogens (carcinogenic in humans)(2,3). Of several aflatoxin producing species, *Aspergillus flavus* and *Aspergillus parasiticus* commonly infect cereal grains including maize, as well as ground and tree nuts. Many of these commodities are important human food staples and are also used in animal feeds. If the grains become infected with *Aspergillus,* aflatoxin contamination may result.

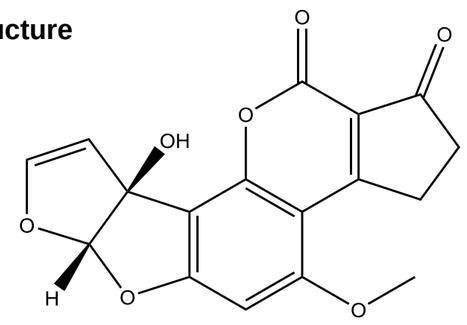
Aflatoxin M1(AFM1) is the focus of this study. It is the 4-hydroxylated metabolite of the most biologically active aflatoxin, aflatoxin B1 (Figure 1) AFM1 can be present in dairy products if animals have consumed aflatoxin contaminated feeds(4). Like it's precursor AFM1 is listed by the IARC as a group 1 human carcinogen(5).

EU regulations for AFM1

- In milk based products sold for adult consumption is 0.050 µg/kg (50 ppt).
- In infant formula, follow-on milk and milk products used in medical settings is 0.025 µg/kg (25 ppt.)(6).

The goal of the following work was to develop a sample cleanup and analysis method facilitating the detection and quantification of AFM1 at the lowest current EU regulatory limit and within EU specified performance criteria(7).

Figure 1. Aflatoxin M1 Structure



Experimental

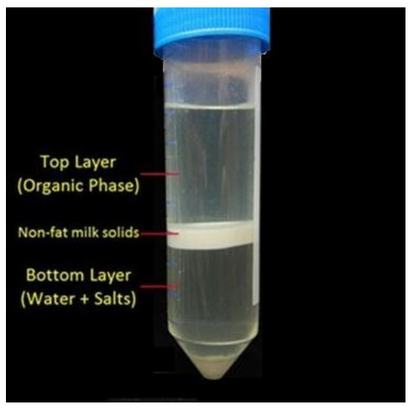
Milk Preparation

1. Weigh 45 grams of milk into 50 mL polypropylene centrifuge tubes.
2. Centrifuge at 5000 rpms (RCF 4863 x G) 60 minutes.
3. Chill milk at 0 °C for 30 minutes.
4. Remove fat (top layer) from milk with laboratory spatula.
5. Transfer 20 mL of milk serum (middle layer) from each processed 45 gram sample to sample collection vessel.

Protein Crash and Solvent Partitioning

1. Pipette 20 mL of acetonitrile into clean 50 mL poly centrifuge tubes.
2. Pipette 20 mL of prepared milk sample into each tube containing acetonitrile. Briefly agitate by hand.
3. Add contents of one Supel™ QuE non-buffered tube #2 (Cat. No. 55295-U) to each mixture. Immediately shake mixture vigorously by hand to disperse salts.
4. Place tubes on shaker at high speed for 5 minutes.
5. Centrifuge shaken tubes @ 5000 rpm (RCF 4863 x G) for 20 minutes.
6. Centrifuged extract will have three layers. The top (organic) layer is the sample layer (Figure 2).

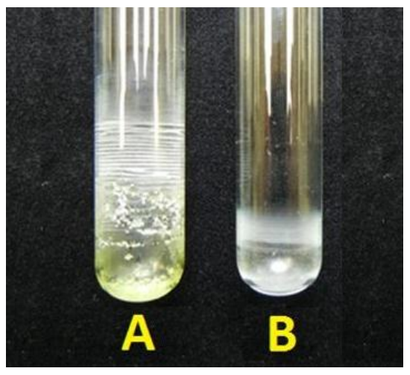
Figure 2. Centrifuged Milk AFM1 Extract



Sample Cleanup

1. Set up 16 x 125 mm silane treated glass tubes for sample collection in Visiprep® vacuum manifold.
2. Pass 10 mL of extract through each SPE tube (Supel™ Tox AflaZea, 55314-U).
3. Immediately follow each sample application with two, 2 mL rinses using acetonitrile (Sample and rinses are collected together for evaporation)
4. Evaporate samples at 70 °C under nitrogen stream.
5. Reconstitute samples in 500 µL of 20:80 acetonitrile: water by vortex mixing for 30 seconds.
6. Filter samples using 13 mm, 0.2 µm PVDF filters and collected into 750 µL polypropylene vials.

Figure 3. Evaporated Milk AFM1 Extraction after: "A" No SPE, "B" AlfaZea SPE



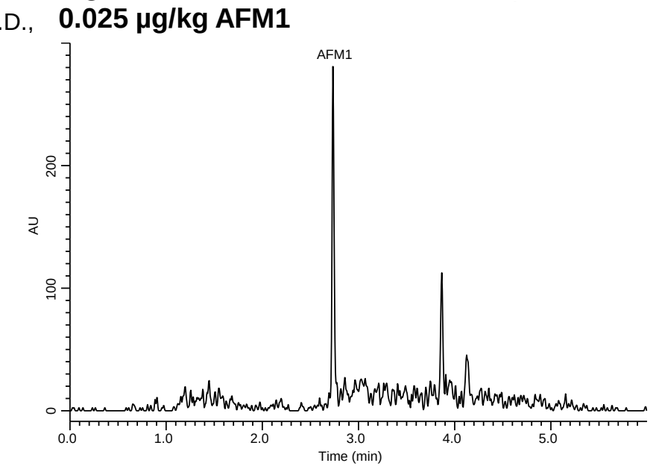
LC-MS Method

column:	Ascentis® Express C18, 50 x 2.1 mm I.D., 2.7 µm
analysis:	LC-MS/MS (329.2→ 273.2)
mobile phase:	(A) 0.1% formic acid, (B) acetonitrile
flow rate:	0.40 mL/min
temp.:	ambient
injection:	10 µL
instrument:	Agilent® 1200/Applied Biosystems 3200 Q Trap

gradient:

Min	%A	%B
0.00	90	10
0.50	90	10
1.00	10	90
4.00	10	90
4.10	90	10
7.00	90	10

Figure 4. Matrix Matched Standard, 0.025 µg/kg AFM1



Quantitation was performed using matrix matched calibration standards ranging from extracted concentrations of 12.5 pg/mL to 500 pg/mL (final concentrations of 250 pg/mL to 10,000 g/mL).

Results

Table 1. Recovery from Milk

	Spiking Levels	
%Recovery (n=3)	0.250 µg/kg	0.025 µg/kg
Ave.	76%	103%
RSD	8.6%	10.2%

Figure 5. AFM1 Fortified Milk Samples 0.25 µg/kg

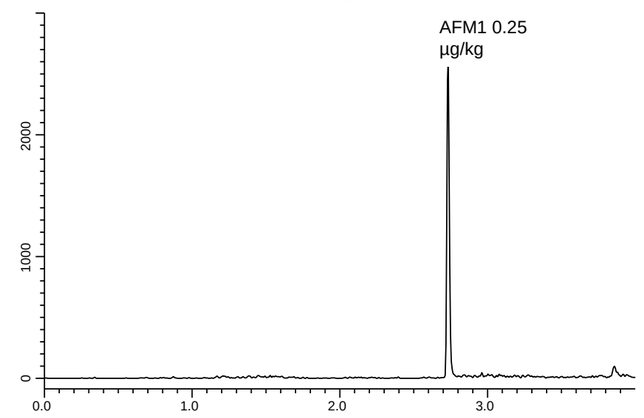
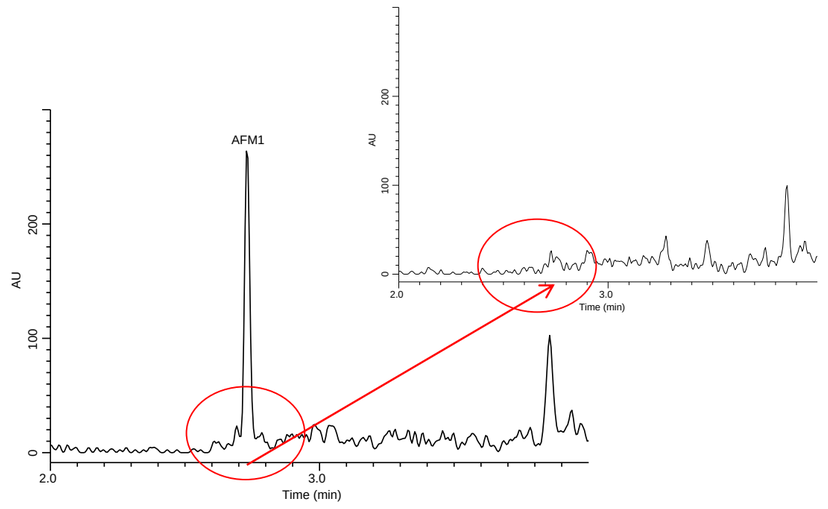


Figure 6. 0.025 µg/kg AFM1 Fortified Sample and a Blank Sample



Discussion

Silane treated glass and polypropylene vials were used to prevent possible binding of AFM1 to untreated glass surfaces.

SPE cleanup using Supel Tox AflaZea was fast and efficient. Conditioning, equilibration and elution steps were not required. Samples passed through the cartridge followed by a simple rinse to ensure a maximum recovery of AFM1.

AFM1 peak was well resolved in LC: There was no significant background interference with the compound of interest when analyzing AFM1 at the low concentration of 0.025 µg/kg (25 ppt.)

During LC-MS analysis some matrix suppression was present in the samples at the retention time of the AFM1 peak. The matrix suppression effect was measured at 40%.

The use of Matrix-matched calibration standards was required due to sample matrix effects.

Using Ascentis Express column provided short HPLC analysis time. The total run time for the LC-MS analysis including a required column wash, was 7 minutes.

Conclusions

The sample preparation procedure developed in this application facilitates the extraction and analysis of low trace (0.025-0.3 µg/kg) concentrations of AFM1 from milk, and appears to be robust with 10% RSD values or less.

Sample recoveries were good

- 76% for 0.250 µg/kg spiked samples
- 103% for 0.025 µg/kg spiked sample
- within the ranges for recovery and precision required by European Union specifications for its treaty members, which are among the most stringent requirements currently written(6,7).

The use of interference removal SPE (Supel Tox AflaZea) cleanup was rapid, less than 5 minutes for 6 samples.

The procedure produced sample cleanliness that was acceptable for LC-MS/MS analysis.

The LC-MS analysis used superficially porous HPLC column (Ascentis Express C18) and was also fast with a run time of 7 minutes.

References

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