Detection of Aflatoxins in Milk at Picogram Levels Using SPE and LC-MS/MS

UCT Part Numbers:
ECHLD126-P – EnviroClean® HL DVB , 200 mg/6 mL SPE cartridge
SLC-18100ID21-3UM – Selectra® C18, 100 × 2.1 mm, 3 µm HPLC column
SLC-18GDC20-3UM – Selectra® C18, 10 × 2.0 mm, 3 µm guard cartridge
SLGRDHLDR – Guard cartridge holder

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Summary:

Aflatoxins are naturally occurring mycotoxins that are produced by several species of fungi (*Aspergillus flavus* and *Aspergillus parasiticus*). They are classified by the International Agency for Research on Cancer as group 1 carcinogens (compounds known to be carcinogenic in humans) [1]. Aflatoxins can occur in food products as a result of fungal contamination of crops (prior to harvest or during storage). There are approximately 20 related aflatoxin metabolites, although only B1, B2, G1 and G2 are normally found in food [2]. Of these, aflatoxin B1 is the most biologically active and most commonly encountered [3].

Aflatoxins can occur in milk as a result of dairy animals consuming contaminated feed. The main residue of concern in milk is aflatoxin M1, the major metabolite of B1. The intake of contaminated milk, even at low concentrations, is a significant threat to human health, especially to children who are a major consumer of dairy products. Therefore, the US Food and Drug Administration has established a tolerance of 0.50 µg/kg for aflatoxin M1 in milk [4], while the European Union has imposed more stringent limits - 0.050 µg/kg in raw milk and 0.025 µg/kg in infant formula [5]. No limits have been established for aflatoxin B1, B2, G1 and G2 in milk.

This application note outlines a method for the low level determination of aflatoxins in milk using a polymeric solid-phase extraction (SPE) cartridge. Analysis is performed by LC-MS/MS using a Selectra® DA HPLC column. The method was optimized to allow the detection of aflatoxins at the low regulatory concentrations required. Recovery studies were carried out by spiking whole milk at two concentration levels (0.025 and 0.5 µg/kg). Matrix-matched calibration curves, ranging from 0.01-2 µg/kg, were used for quantitation. The mean recovery was found to be in the range of 84 to 100%, and repeatability was ≤7%.

Procedure:

Aflatoxins are relatively unstable in light and air, particularly in polar solvents or when exposed to oxidizing agents, ultraviolet light, or solutions with a pH below 3 or above 10. They should be protected from ultraviolet light as much as possible.

1. **Sample extraction 1 (aqueous extraction)**
   a) Weigh 20 g of milk into a 50 mL polypropylene centrifuge tube.
   b) Add 200 µL of glacial acetic acid.
   c) Vortex for 5 minutes to deproteinize the milk.
   d) Centrifuge for 5 minutes at ≥ 4000 *g*.

2. **SPE extraction**
   a) Condition SPE cartridge with:
      1. 1 × 3 mL methanol
      2. 1 × 3 mL ultrapure water
   b) Apply the supernatant to the SPE cartridge, taking care to avoid any transfer of the lipid layer. If required, use a low vacuum to draw the sample through (≤5 mL/min).

3. **Sample extraction 2 (solvent extraction)**
   a) Add 10 mL acetone to any residual milk solids from the aqueous extraction.
   b) Vortex for 2 minutes to extract the aflatoxins.
   c) Centrifuge for 5 minutes at ≥ 4000 *g*.
   d) Transfer the supernatant to a clean polypropylene tube and evaporate to ≤0.5 mL at 50°C under a gentle stream of nitrogen.
   e) Add 10 mL ultrapure water and vortex briefly.
   f) Apply sample to the SPE cartridge (same cartridge as step B).

4. **Wash cartridge**
   a) 1 × 3 mL ultrapure water.
   b) 1 × 3 mL 50% methanol.
   c) Dry cartridge under vacuum (≥10 inHg) for 5-10 minutes to remove residual water.
   d) 1 × 3 mL hexane.
   e) Dry cartridge under vacuum (≥10 inHg) for 1 minute to remove residual hexane.

5. **Elution**
   a) Elute the aflatoxins with 4 mL acetone.
   b) Evaporate the sample to dryness at 50°C under a gentle stream of nitrogen.
   c) Reconstitute in 1 mL of methanol:water (50:50, v/v).
   d) Filter extract with a 0.22 µm nylon (or other suitable membrane) syringe filter into an autosampler vial.

LC-MS/MS Conditions:

HPLC Conditions	
Instrumentation	Thermo Scientific™ Dionex™ Ultimate™ 3000
HPLC column	UCT Selectra® C18, 100 × 2.1 mm, 3 µm **(p/n: SLC-18100ID21-3UM)**
Guard column	UCT Selectra® C18, 10 × 2.0 mm, 3 µm **(p/n: SLC-18GDC20-3UM)**
Guard column holder	**p/n: SLGRDHLDR**
Column temp.	40°C
Mobile phase A	Water + 0.1% formic acid
Mobile phase B	Acetonitrile + 0.1% formic acid
Flow rate	300 µL/min
Gradient	0 min (5% B), 5-6 min (hold 100% B), 6.1-11 min (equilibrate 5% B)
Injection volume	20 µL
Autosampler temp.	10°C
Wash solvent	Methanol
Divert valve	Divert to waste at 0-4 and 6-11 min to reduce ion source contamination

MS Conditions	
Instrumentation	Thermo Scientific™ TSQ
Ionization mode	ESI^+
Spray voltage	3500 V
Vaporizer	400°C
Capillary	350°C
Sheath gas pressure	55 arbitrary units
Auxiliary gas	45 arbitrary units
Ion sweep gas	0 arbitrary units
Declustering	0 V
Q1 and Q3 peak	0.2 and 0.7 Da
Collision gas	Argon
Collision gas	2.2 mTorr
Acquisition method	EZ method (scheduled SRM)
Cycle time	0.6 sec
Software	Xcalibur™ version 2.2

SRM Transitions							
Analyte	**t_R (min)**	**Precursor ion**	**Product ion 1**	**CE 1**	**Product ion 2**	**CE 2**	**S-lens (V)**
Aflatoxin M1	4.7	329.0	273.0	21	259.0	23	120
Aflatoxin G2	4.9	331.0	245.0	27	189.0	38	115
Aflatoxin G1	5.0	329.0	243.0	25	199.0	46	117
Aflatoxin B2	5.0	315.0	287.0	24	259.0	27	111
Aflatoxin B1	5.2	313.0	241.0	35	285.0	21	111

Results and Discussion:

Accuracy & Precision Data for Whole Milk				
	0.025 µg/kg (n=5)		0.5 µg/kg (n=5)	
	Mean Recovery (%)	RSD (%)	Mean Recovery (%)	RSD (%)
Aflatoxin M1	94.43	3.54	90.91	4.31
Aflatoxin B1	89.82	4.19	84.34	4.31
Aflatoxin B2	93.27	3.76	88.27	7.51
Aflatoxin G1	92.51	5.48	89.28	7.06
Aflatoxin G2	100.05	1.71	93.51	6.83

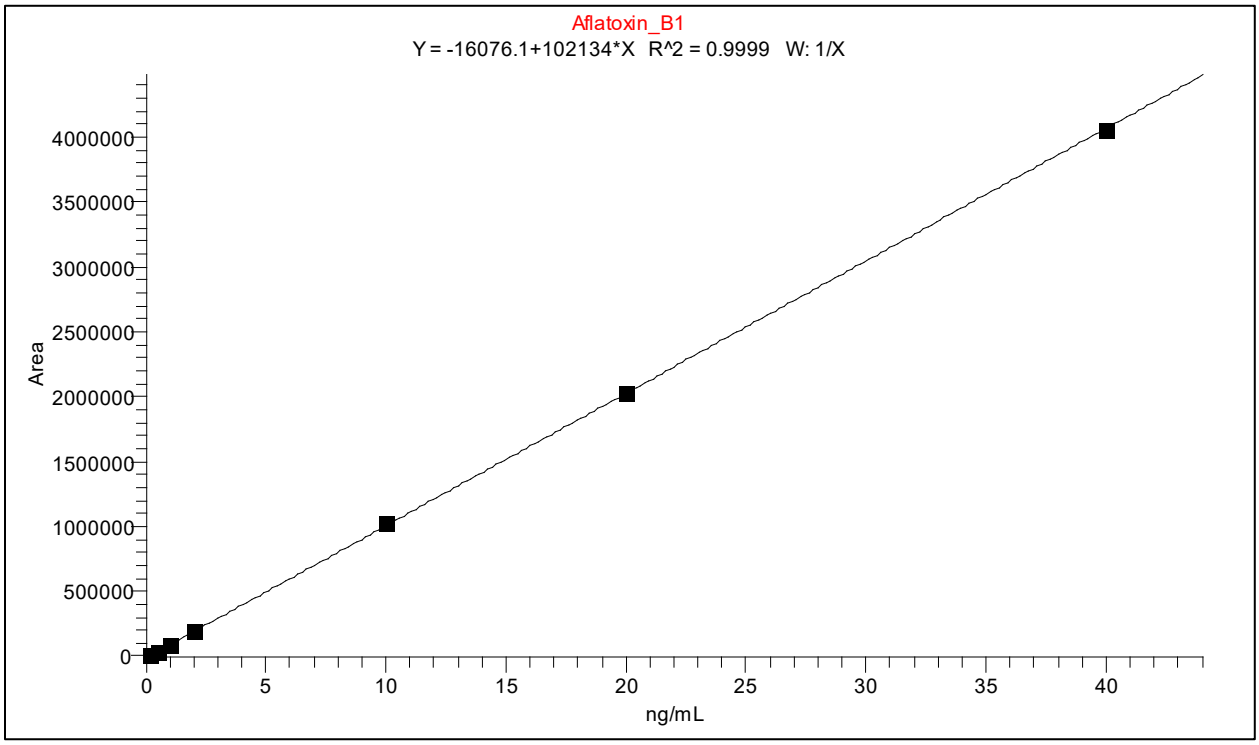

Figure 1. Example of a seven point matrix-matched calibration curve (0.2, 0.5, 1, 2, 10, 20 and 40 ng/mL; equivalent to 0.01, 0.025, 0.05, 0.1, 0.5, 1 and 2 µg/kg in milk).

Chromatograms

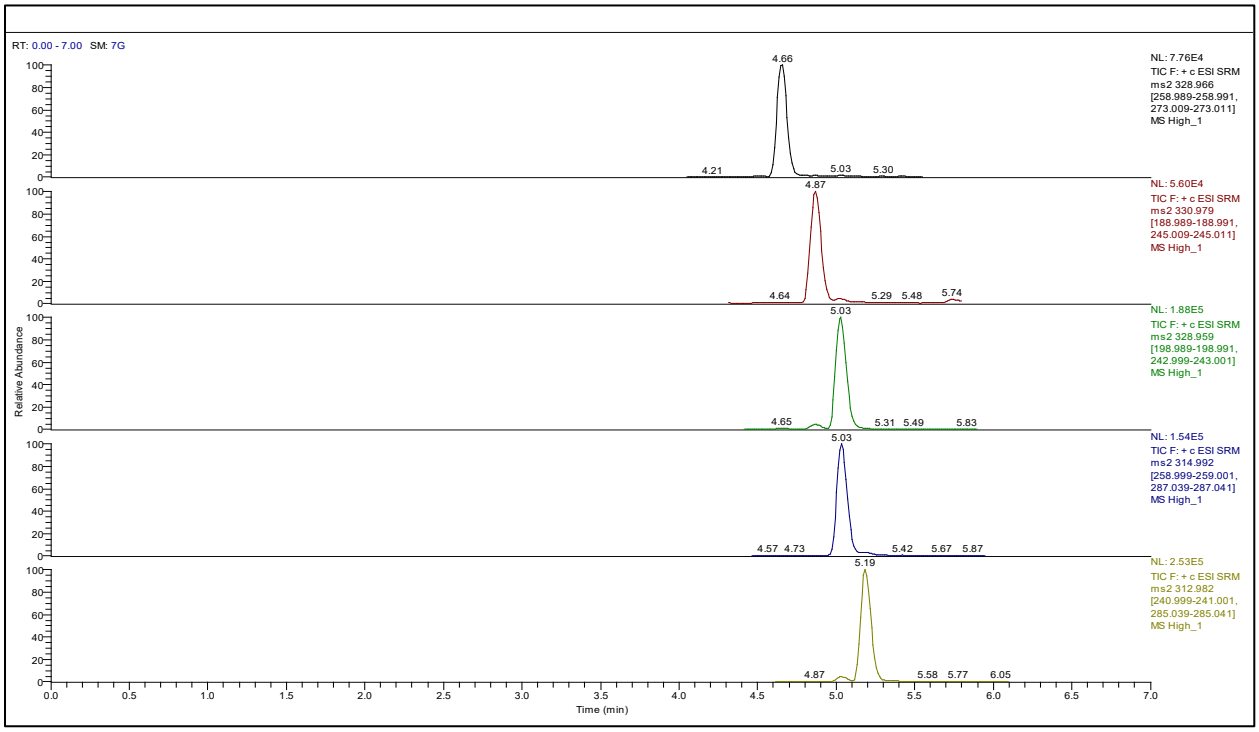

Figure 2. Chromatogram of an extracted milk sample fortified at 0.5 µg/kg.

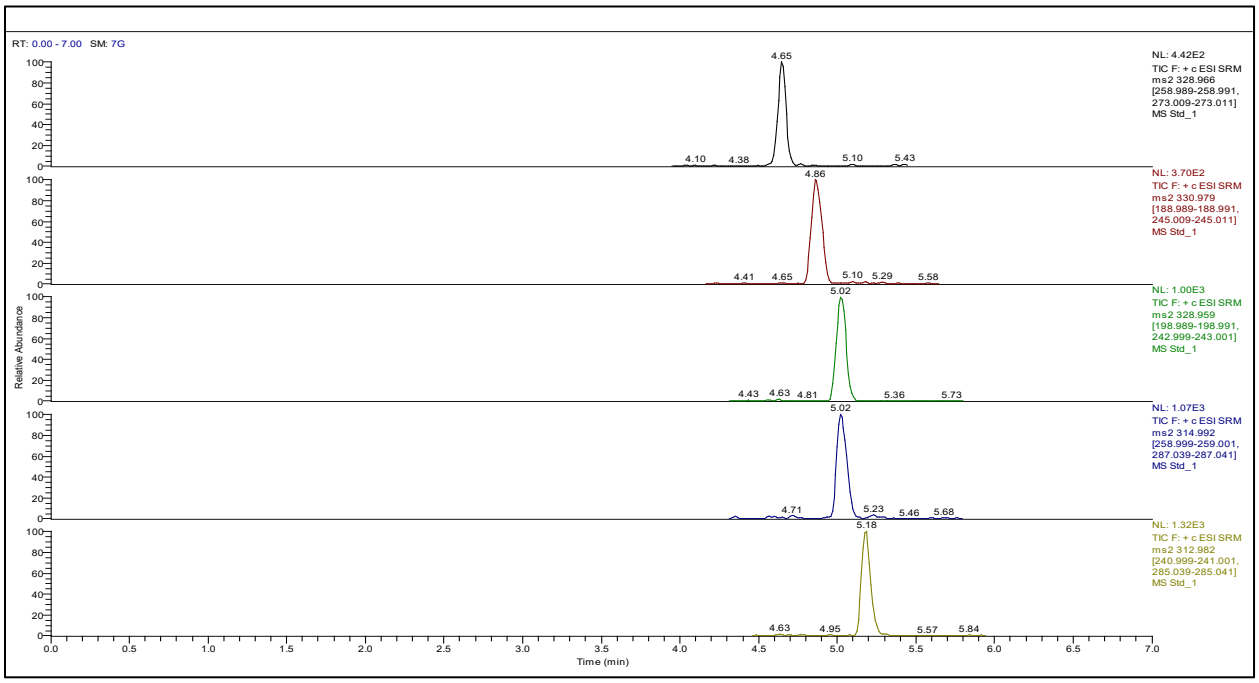

Figure 3. Chromatogram of the lowest matrix-matched calibration point (0.01 µg/kg).

Prior to instrumental analysis, a sample pre-treatment step is required to concentrate the analyte(s) of interest and eliminate non-desirable matrix components. This is particularly important for the analysis of aflatoxins in milk because of the low regulatory limits established for aflatoxin M1 and the larger sample size required to obtain the necessary method sensitivity. SPE is ideally suited to achieving these objectives. One of the biggest difficulties in milk analysis is the high fat and protein content that can often interfere with instrumental analysis. The sample preparation procedure was therefore optimized to remove as much co-extracted matrix components as possible.

Initially, a simple deproteinization step using acetic acid followed by centrifugation was executed to separate the proteins and lipids prior to SPE extraction. However, the recoveries were found to be low (<40%) using this approach, which is most likely caused by the adsorption of the aflatoxins onto proteins or lipids in the milk. It was determined that a solvent extraction step was necessary to adequately extract the aflatoxin residues from milk prior to SPE cleanup. Due to the high water content of milk, direct extraction with an organic solvent would result in a large volume of supernatant that could not be directly applied to the SPE cartridge (organic content too high) or require a time consuming evaporation step to remove the solvent. As a result, a two-step extraction procedure incorporating an initial aqueous extraction step was included in the final method. This simple step removes most of the water from the sample prior to a second extraction with acetone, a volatile organic solvent that is readily removed by evaporation. This extract is then reconstituted in water prior to application to the SPE cartridge. The SPE sorbent was washed with 50% methanol to remove medium to highly polar matrix components and hexane to remove lipophilic compounds. Acetone was used as the SPE elution solvent as it was found to be more effective than methanol and very easy to remove by evaporation. Filtration of the final sample extract prior to LC-MS/MS analysis and the use of matrix-matched calibration curves and/or isotopically labeled internal standards are recommended to obtain optimal results.

References:

1. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, International Agency for Research on Cancer (IARC) website, http://monographs.iarc.fr/ENG/Classification/index.php (accessed June 2015).
2. European Mycotoxins Awareness Network website, http://services.leatherheadfood.com/eman/FactSheet.aspx?ID=6 (accessed June 2015).
3. European Food Safety Authority website, http://www.efsa.europa.eu/en/topics/topic/aflatoxins.htm (accessed June 2015)
4. FDA Compliance Policy Guide, Sec. 527.400 Whole Milk, Lowfat Milk, Skim Milk - Aflatoxin M1.
5. Commission Regulation (EU) No 165/2010. Amending Regulation (EC) No 1881/2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs as Regards Aflatoxins. *Official Journal of the European Union*, Feb 26, 2010, pp L 50/8 – L 50/12.

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