Quantification of aflatoxin M_1 in milk using the SCIEX QTRAP® 4500 LC-MS/MS System

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Milk is a very healthy, nutritious food, full of key nutrients for growth. In 2018, about 66.8 million metric tons of milk was consumed in India and an average of 30 million metric tons of milk was consumed in Europe and United states. One of the major forms of contamination that can be found in milk are mycotoxins, which are toxic compounds produced by certain fungi that can have serious health effects even at very low concentrations.[1,2] Regular consumption of aflatoxin M_1 in children may cause stunted growth, cirrhosis and cancer. Considering its serious effects in pediatrics, every government has set up stringent monitoring of aflatoxin M_1 in milk and its products. India as well has thus tightened the inspection of the toxins in milk samples. However, the determination of this mycotoxin in the milk samples is very difficult due to the complexity of the matrix (fat, proteins, carbohydrates, etc.).

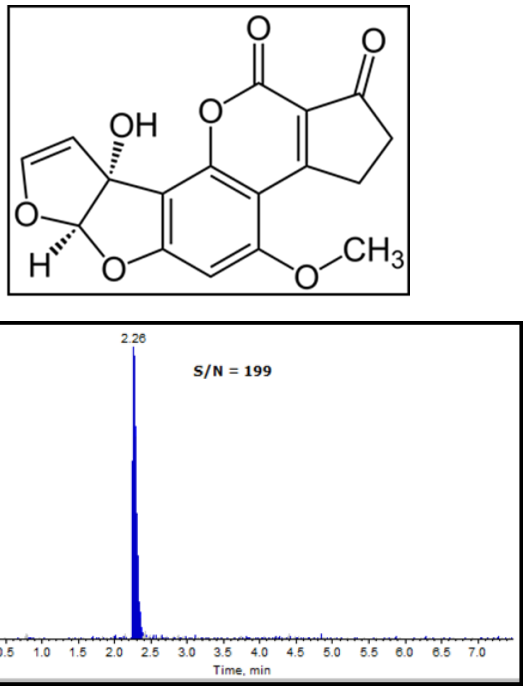


Figure 1: Structure of aflatoxin M_1. (Top) Aflatoxin M_1 is a chemical compound belonging to a group of mycotoxins produced by aspergillus. $C_{17}H_{12}O_7$, MW: 328.276 g/mol. (Bottom) Chromatogram showing S/N ratio of aflatoxin M_1 at MRL level (0.50 ppb). concentration.

A robust method was developed in the SCIEX QTRAP 4500 System coupled with the ExionLC™ System for the targeted quantification of aflatoxin M_1 in milk samples.

Key features of targeted quantification method for aflatoxin M_1

- A targeted quantitative method has been developed on the SCIEX QTRAP 4500 System using two MRM transitions
- A simple sample preparation method was developed for extraction of milk samples
- Sensitivity was easily sufficient to meet the MRL requirements (0.5 ppb) for aflatoxin M_1 in milk
- The developed method was validated as per the regulatory guidelines described in Commission Decision (2002/657/EC) directive[3]

Methods

Sample preparation: Standard aflatoxin M_1 was purchased from Sigma Aldrich. All other chemicals used were of LC-MS grade, commercially available. Milk samples were purchased from local vendors and were stored in refrigerator at 2 to 8 °C until sample analysis.

An optimized extraction procedure was developed in which 5 mL of milk was mixed with 10 mL of acetonitrile and vortexed for 10 mins. 2 g of NaCl was added, vortexed for 5 min and centrifuged at 3200 rpm for 5 min at 4 °C. The organic layer was collected and evaporated to dryness under nitrogen. The residue was reconstituted for 1 mL with water/methanol (90:10), filtered and transferred into autosampler vials for LC-MS/MS analysis.

Chromatography: Separation was performed using an ExionLC™ System using Kinetex 1.7 µm C18 column (100°A, Size: 150 x 2.1 mm). An injection volume of 20 µL was used. The gradient program is shown in Table.1.

Table 1. Gradient profile and mobile phase composition.

Total Time (min)	Flow Rate (µL/min)	A%	B%
0.00	*500*	*90*	*10*
4.00	*500*	*5*	*95*
4.10	*500*	*90*	*10*
7.50	*500*	*90*	*10*

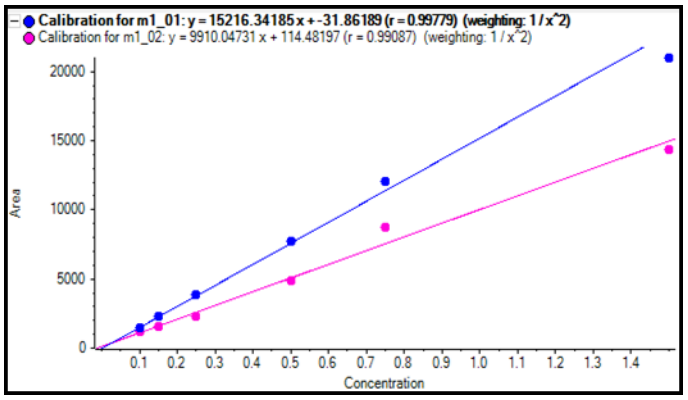
Mobile phase A: water + 0.1% formic acid
Mobile phase B: acetonitrile + 0.1% formic acid

Mass spectrometry: The SCIEX QTRAP® 4500 LC-MS/MS System was operated in multiple reaction monitoring (MRM) mode. The Turbo V™ Ion Source was used with an electrospray ionization (ESI) probe, in positive polarity at an ion spray voltage of 2800. The MRM transitions used are shown in Table 2. Analyst® Software 1.7 was used for method development and data acquisition.

Data processing: LC-MS/MS data was processed using the MultiQuant™ Software 3.0.3.

Table 2. MRM transitions of aflatoxin M_1.

Compound	Precursor Ion	Product Ion (Quantifier)	Product Ion (Qualifier)	Collision Energy (CE)
Aflatoxin M_1	*329.1*	*259.1*	*273.0*	*51 / 44*

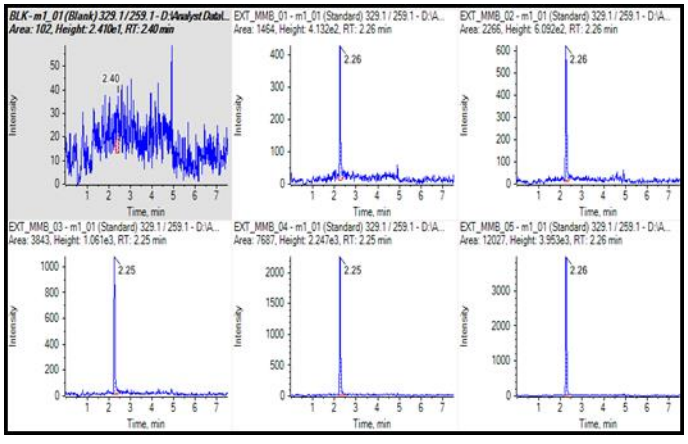


Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV
m1_01	0.15	10 of 10	2.630e3	6.692e1	2.54

Figure 2: Calibration curve of aflatoxin M_1 in milk. Good linearity was achieved for the concentration range evaluated (0.15 to 1.50 ppb) with r values >0.99 for both MRM transitions, with $1/x^2$ weighting. Good reproducibility data was observed for aflatoxin M_1 in extracted samples at the LOD level of 0.15ppb, 2.54 %CV for 6 replicates.

Results

First, the chromatography was optimized to provide a fast 7.5 min run time (Figure 1). Calibration curves were run across a concentration range of 0.15 to 1.50 ppb of aflatoxin M_1 in extracted milk matrix to determine the performance of the method. A LOD of 0.15 ppb was achieved in extracted milk and the %CV (n=10) was still <5%. Good linearity across the range was observed (Figure 2). Reproducibility was evaluated for both inter and intra days with the replicates of six injection and the %CV was found to be <5%, which proves the method is rugged and reproduceable below its MRL level (0.50 ppb) (Figure 3,



Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV
m1_01	0.50	6 of 6	9.182e3	3.671e2	4.00

Figure 3: Representative chromatograms of aflatoxin M_1. The integrated peaks for aflatoxin M_1 from 0.15 ppb to 1.5 ppb are shown here, including the blank.

bottom). No significant matrix interferences were observed (Figure 3, top). Ion ratios were used to ensure that the correct peaks were integrated in each sample, for added confidence in detection (Figure 4).

Conclusions

The method developed here enables the fast and accurate quantification and confirmation of aflatoxin M_1 in extracted milk samples by LC-MS/MS. The SCIEX QTRAP 4500 System provides good sensitivity and selectivity for this analysis, with minimal sample preparation, providing maximized throughput for the analysis of large sample batches in a short time period. The method was developed in accordance with Commission Decision (2002/657/EC) directive recommendations and showed acceptable accuracies (70%-130%) across the calibration curves in matrix, had good linearity for both the MRM transitions and had high repeatability with %CV < 5%.

This rapid and sensitive LC-MS/MS method for the quantification of aflatoxin M_1 in milk also easily meets the MRL of 0.5 ppb level for this toxin in milk.

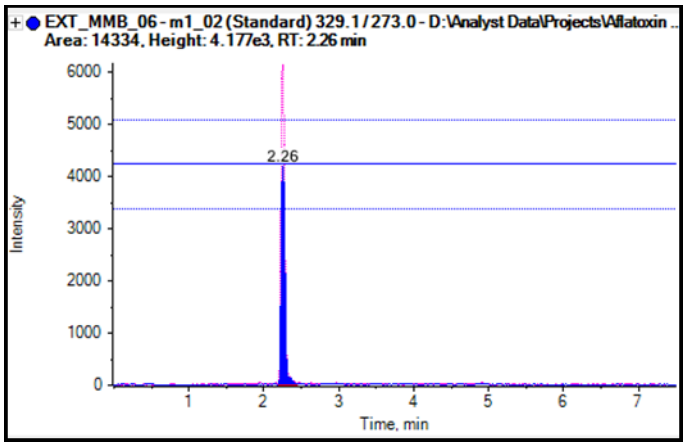


Figure 4: Representation of the aflatoxin M_1, with 20% difference in the ion ratio.

References

1. Zhang K *et al.* (2017) Determination of Mycotoxins in Corn, Peanut Butter, and Wheat Flour Using Stable Isotope Dilution Assay (SIDA) and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). *J. Agric. Food Chem.* **65(33)**, 7138-7152.
2. Carcinogen Aflatoxin detected in FSSAI milk survey samples. (2019)
3. EC European Commission, Commission Decision 2002/657/EC of 12 August 2002. Off. J Eur. Communities, L221, 8–36.

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