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DEVELOPMENT OF METHOD FOR THE DETERMINATION OF AFLATOXIN M1 IN RAW MILK AND MILK POWDER BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LCMSMS)

M. MATIUR RAHIM[1]*, M. TARIQUL HASSAN[1], M. NURUL HUDA BHUIYAN[1], MAKSUDA BEGUM[1] and M. ANWAR HOSSAIN[2]

[1]Food Toxicology Research Laboratory, Institute of Food Science & Technology (IFST), Bangladesh Council for Scientific and Industrial Research (BCSIR), Dhaka-1205 and [2]Process Engineer, Chemicals Incorporated, Baytown, Texas, USA. *Corresponding author's Email: matiurrahim@yahoo.com

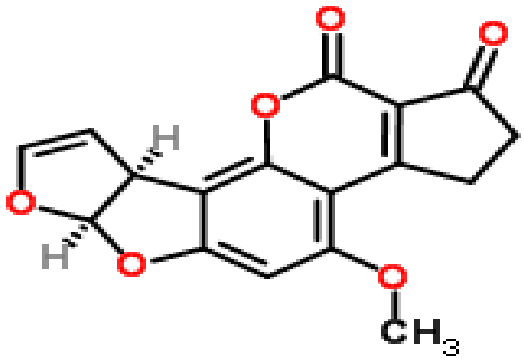
ABSTRACT

The study was conducted during 2011-2013 at the Food Toxicology Laboratory of IFST, Bangladesh Council of Scientific and Industrial Research, Dhaka, Bangladesh under an R&D project entitled "Occurrence of aflatoxin M1 in milk and dairy products available in the markets of Bangladesh". Aflatoxin M1(AFM1) is a hepatocarcinogen found in milk of animals that have consumed feeds contaminated with aflatoxin B1(AFB1), the most important toxic metabolite produced by storage fungi of the genus *Aspergillus*, particularly *Aspergillus flavus* and *Aspergillus parasiticus*. This procedure describes the extraction of Aflatoxin M1 from milk samples using a Phenyl solid phase extraction (SPE) procedure followed by liquid chromatography tandem mass spectrometry (LCMSMS) analysis. The method allows for superior clean up and selective detection with a detection limit of 12.50 ng/L.

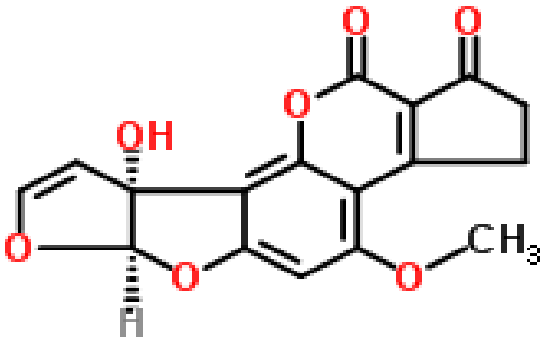
Keywords: Aflatoxin M1, Raw milk, Powder milk, SPE Clean-up and LCMSMS method.

INTRODUCTION

Aflatoxins are a group of naturally occurring toxins produced mainly by moulds named *Aspergillus flavus.* When aflatoxin B1 (AFB1), the most toxic aflatoxin, is ingested, some animals are able to convert it into AFM1, which, in turn, is transferred to milk, eggs or meat (Polan *et al.,* 1974; Trucksess and Stoloff, 1984). The conversion rate of aflatoxin B1 to aflatoxin M1 present in milk ranges between 0.5% and 5% for many mammals including dairy cattle and humans, yet values as high as 6% have been reported by Pipet (Pipet, 1998). AFM1 exhibits carcinogenic (IARC, 2002), genotoxic (Lafont *et al.,* 1989) and cytotoxic effects (Neal *et al.,* 1998).



Aflatoxin B1



Aflatoxin M1

AFB1 and AFM1 have been classified by the International Agency for Research on Cancer as human carcinogens class 1 and 2B respectively (IARC, 2002). Because of their potential risks and also to minimize their hazard, the World Health Organization recommends the reduction in its consumption to a minimum, because there still is not enough information available to establish a tolerable exposure level (WHO, 2002).

So, after the development of Aflatoxin M1 analysis method we can help the dairy industries to produce value added dairy products which will contribute towards the export/import trade of the country and also can help the people to consume aflatoxin M_1 free milk and dairy products.

MATERIALS AND METHOD

The study was conducted at the Food Toxicology Laboratory of IFST, Bangladesh Council of Scientific and Industrial Research, Dhaka, Bangladesh during 2011-2013 under an R&D project entitled "Occurrence of aflatoxin M1 in milk and dairy products available in the markets of Bangladesh".

Fluid milk: Measure 30ml fluid milk into a 50 ml polypropylene centrifuge tube. Centrifuge the sample at 2000 rpm for 15 minutes. Separate the fat (top) layer from the defatted (skim) milk. Use 20ml of the defatted milk for further analysis and filter the 20ml defatted milk through a 0.45µm filter prior to loading onto the SPE column.

Milk powder: Weigh 5g milk powder. Heat 50 ml purified water to 60°C. Add 30 ml preheated water to the milk powder. Stir for 10 minutes. Transfer to a measuring cylinder and bring the volume to 50 ml with the remaining preheated water. Centrifuge the sample at 5000 rpm for 15 minutes. Centrifuge the sample at 2000 rpm for 15 minutes. Separate the fat (top) layer from the defatted (skim) milk. Use 20ml of the defatted milk for further analysis and continue the analysis as for fluid milk.

Instrument

HPLC: Pump – Shimadzu LC20AD
Column: Luna PFP (2) C18 2.0 x 50mm, 5µm
Flow rate: 0.50 ml/minute
Injection volume: 20µl
Column temperature: 35°C
Pressure range: 0.0 – 40.0 MPa
Mobile phase: i. Water containing 0.1% formic acid and ii. Acetonitrile containing 0.1 % formic acid.

Gradient elution:
10% B for 1.5 minute → 95% B at 4 minutes → Hold for 4 minutes
10% B at 8.01 minutes → Hold for 2 minutes. Run time 10 minutes.

MS: Spectrometer: 3200 Q TRAP (Applied Biosystems)

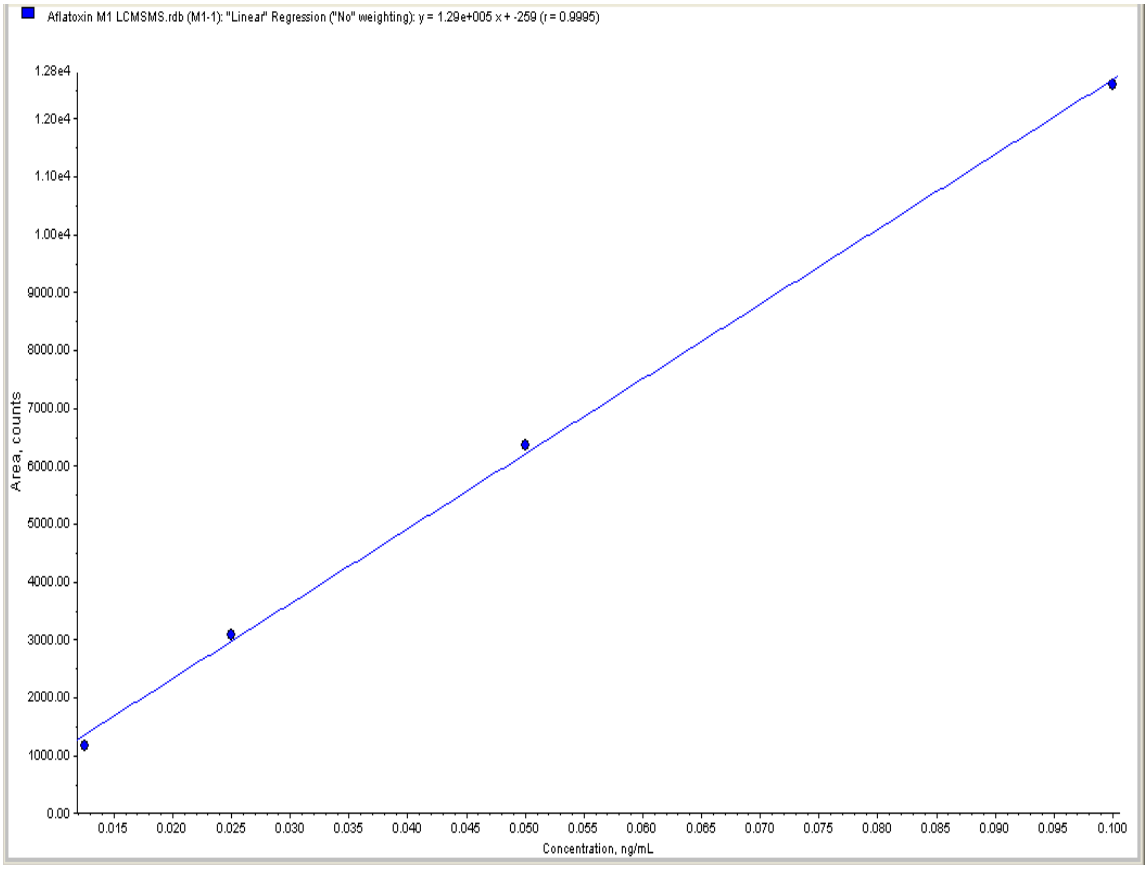
Source conditions: CUR: 15, IS: 5000V, GS1: 50, GS2: 50, TEM: 550°C
Acquisition mode: Multiple reactions monitoring (MRM) (Table 1).

Table 1. MRM's.

Compound	Ion source	Ionisation mode	Precursor ion (m/z)	Product ions (m/z)
Aflatoxin M1	Turbo Spray	Positive	329.06	273.20 & 229.10

RESULT AND DISCUSSION

The level of AFM1 in milk should not exceed 500 pg ml-1(i.e. 0.5µg/kg) according to US regulations but the level is set at 50 pg ml-1(i.e. 0.05µg/kg) in most European countries and in the *Codex Alimentarius* (Codex Alimentarius Commission, 2001). The maximum permitted levels allowed in the European Union for Aflatoxin M1 is set out in commission regulation (EU) No. 165/2010 of 26 February 2010. This is set at 0.050µg/kg for raw milk, heat-treated milk and milk for the manufacture of milk-based products. The maximum permitted level is 0.0250µg/kg for infant formulae and follow-on formulae, including infant milk and follow-on milk. In Bangladesh, the action levels have not been officially set for AFM1 in milk yet. So, in this study we have developed our method for the analysis of Aflatoxin M1 at the level of 0.0125µg/kg to 0.1µg/kg linearity range (Fig. 1) so that we can easily quantify Aflatoxin M1 at its non-permissible level.



Concentration, ng/ml (i.e. µg/kg)

Fig. 1. Linearity Curve for Aflatoxin M1 Analysis using LCMSMS.

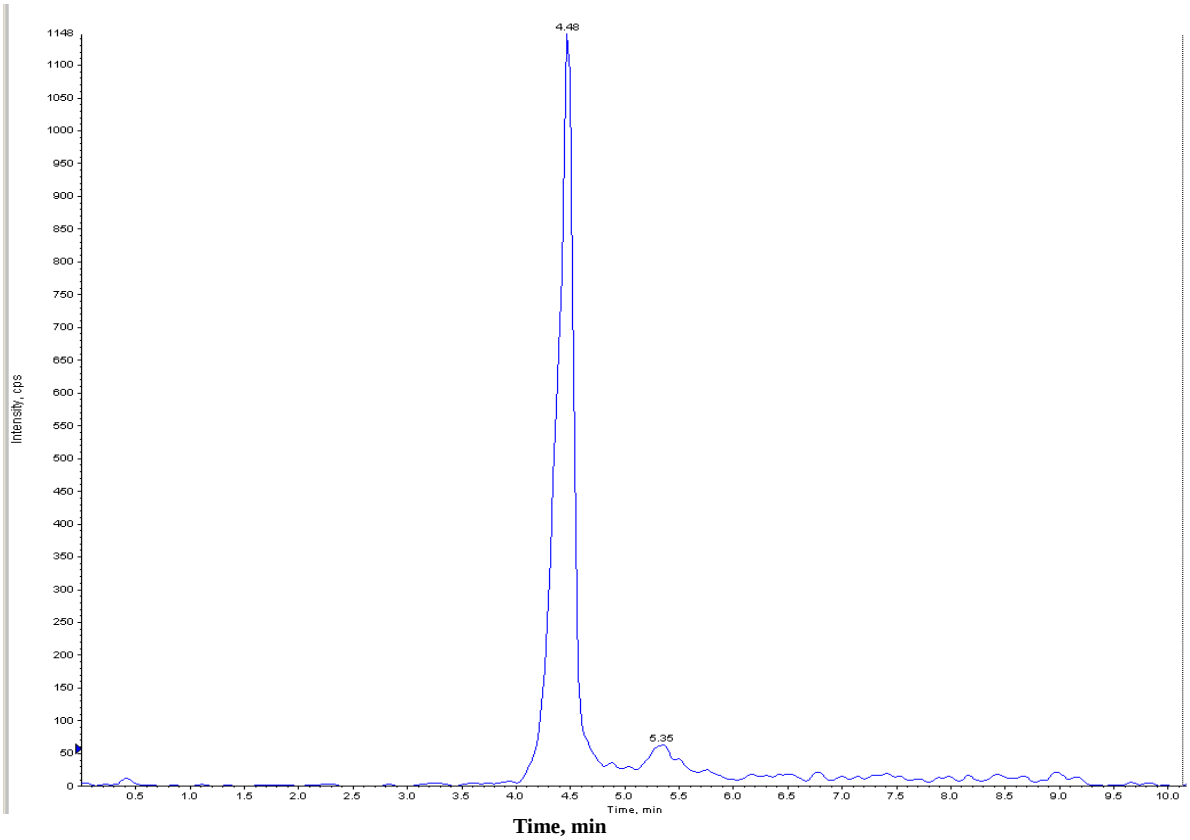


Fig. 2. Chromatogram of 0.1 ppb (µg/kg) Aflatoxin M1 spiked milk sample.

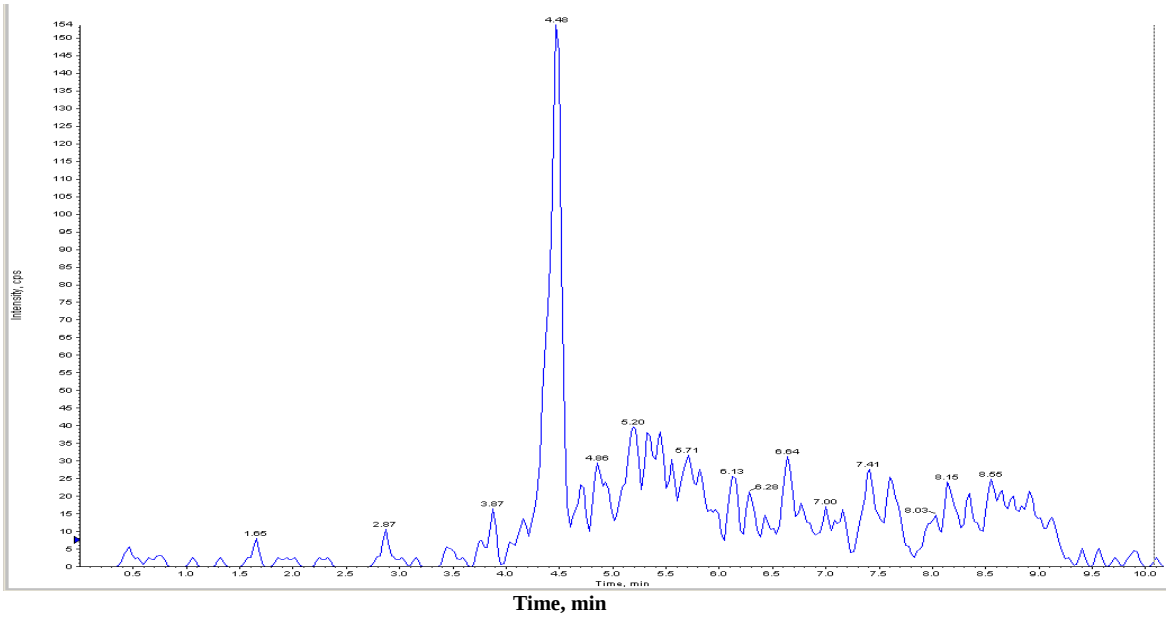


Fig. 3. Chromatogram of 0.0125 ppb (µg/kg) Aflatoxin M1 spiked milk sample.

The range that was examined for the analysis of Aflatoxin M1 was between 0.0125 (Fig. 3) and 0.1(Fig. 2) µg/kg to accommodate the low maximum permitted levels prescribed by legislation. The recovery at 0.1 ppb was 99.3% and at 0.0125 ppb was 88.6% (Table 2).

Table 2. Calibration table with recovery/Accuracy.

Sl no.	Sample ID	Sample type	File name	Analyte peak area (counts	Analyte peak height (cps)	Analyte concentration (mg/ml)	Use record	Calculated concentration (mg/mL)	Accuracy
1	Aflatoxin M1-01.ppb	Standard	21112013/Data SET2.wiff	1.26e+004	8.95e+002	0.100	√	0.0993	99.3
2	Aflatioxin-M1-0.025	Standard	21112013/Data SET2.wiff	3.08e+003	2.17e+002	0.0250	√	0.0258	103
3	Aflatioxin-M1-0.025ppb	Standard	21112013/Data SET2.wiff	1.17e+003	9.62e+001	0.0125	√	0.0111	88.6
4	Aflatioxin-M1-0.025ppb	Standard	21112013/Data SET1.wiff	6.37e+003	4.35e+002	0.0600	√	0.0612	102

CONCLUSION

As milk is the main nutrient for infants and children who are considered to be more susceptible to adverse effects of mycotoxins, the occurrence of AFM1 in milk is a concern. On the other hand, milk is not only consumed as liquid milk, but also utilized in the preparation of infant formulas, yogurt, cheese and milk-based confectioneries including chocolates and pastry. Therefore, it is important and necessary to determine AFM1 levels in milk and dairy products in order to protect consumers in various age groups, from its potential hazards. For this purpose a sensitive, accurate and precise LC-MS/MS method for the detection of Afl atoxin M1 in milk was developed.

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