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Compensating Matrix Effect and Low Extraction Recoveries by Adopting Procedural Standard Calibration Approach for Quantification of Mycotoxins in Milk Samples

Prasanth Joseph[1]; Rohit Ojha.[1]; Saikat Banerjee[1];
Kannan Balakrishnan[1]

[1]Agilent Technologies, Bangalore, India

Introduction

Commercially sold milk is a dense source of essential macro- and micronutrients. However, it may also be a source of natural food contaminants.

Mycotoxins are produced as secondary metabolites by various fungi species that grow on various crops during storage. If cows ingest contaminated feed, animal's milk can also get contaminated with mycotoxins and their metabolites. Aflatoxin M1 in milk is regulated in many countries. The aflatoxin M1 is a mycotoxin that results from the hydroxylation of the aflatoxin B1. Also, there is a growing interest to monitor additional mycotoxins such as Aflatoxin B1, B2, G1 and G2.

Due to matrix effects by high protein and lipid content, analysis of mycotoxins in milk can be challenging because of poor recovery at low concentration levels. Instead of using internal standard to compensate the recovery loss, an approach based on procedural standard calibration method is developed.

Figure 1: Chemical structures of selected aflatoxins

Experimental

Method Parameters

A multiple reaction monitoring (MRM) method was developed using an Agilent 6470 triple quadrupole LC-MS/MS. Reverse phase gradient program with 0.1% Formic acid and 5 mM ammonium formate in water as mobile phase A and 0.1% Formic acid and 5 mM ammonium formate in Acetonitrile/Methanol (50/50) as mobile phase B in a Poroshell SB C18 (2.1 x 100 mm, 2.7 µm) stationary phase was employed to achieve the chromatographic resolution of analytes.

Time, min	%A	%B
0	80	20
7	30	70
7.25	5	95
11	5	95
Post run	2 min	

Figure 2. Mobile phase gradient timetable

To 10.0 ml milk sample, added 10.0 ml Acetonitrile and mixed well

Extraction of analytes by the added the QuECHERS extraction kit powder (Agilent P/N:5982-5550).

Sample clean up by the dispersive kit (Agilent P/N: 5982-1010). Transfer supernatant to 2ml vial and inject in LCMS.

Figure 3. Flowchart for sample preparation

Compound ID	Product m/z	Res Q1	Product m/z	Res Q2	Frag.	CE	CAV	Ionization
Aflatoxin B1	313	Unit	285.1	Unit	152	28	4	ESI Positive
		Unit	241	Unit		40	4	ESI Positive
Aflatoxin B2	315	Unit	287.1	Unit	156	28	4	ESI Positive
		Unit	243	Unit		44	4	ESI Positive
Aflatoxin G1	329	Unit	243.2	Unit	132	32	4	ESI Positive
		Unit	115.2	Unit		84	4	ESI Positive
Aflatoxin G2	331	Unit	313	Unit	154	24	4	ESI Positive
		Unit	285.1	Unit		28	4	ESI Positive
Aflatoxin M1	329	Unit	128	Unit	116	68	4	ESI Positive
		Unit	301.2	Unit		12	4	ESI Positive

Figure 4. MRM parameters

Separation between five aflatoxins

The optimized chromatography of 11 minutes is followed by 2 minutes of post run with initial conditions of gradient. The retention time of mycotoxin M1, B1, B2, G1 and G2 were at 5.06 minutes, 5.62 minutes, 5.31 minutes, 5.05 minutes, and 4.71 minutes, respectively.

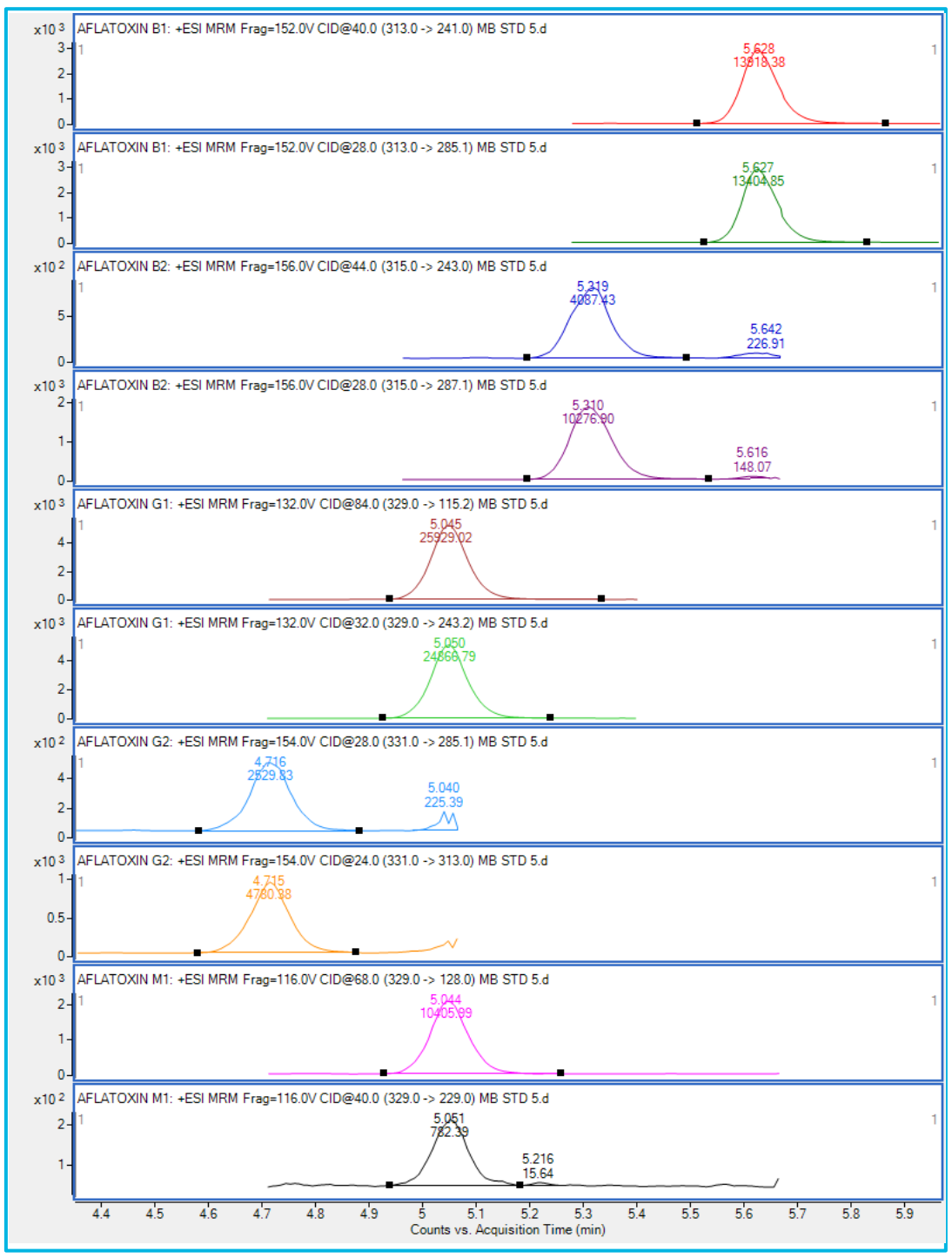

Figure 5: Representative chromatograms of aflatoxin B1, B2, G1, G2 and M1

All five aflatoxins were ionized well in ESI positive mode, and the limit of quantification were 0.5 ng/ml for Afltaoxins-B1, B2, G1 and G2 and 0.05 ng/ml for Aflatoxin M1. Calibration range for Aflatoxin B1, B2, B3 and B4 were between 0.5 to 10 ng/ml. However, for Aflatoxin M1 calibration range was selected between 0.05 ng/ml to 1 ng/ml. Procedural calibration curves were found linear using linear regression and 1/X weighing with regression coefficient above 0.995 for all the five aflatoxins.

Procedural standard calibration curves

Procedural standards are prepared by spiking a series of blank test portions with different amounts of analyte, prior to extraction and then prepared by the same way as samples.

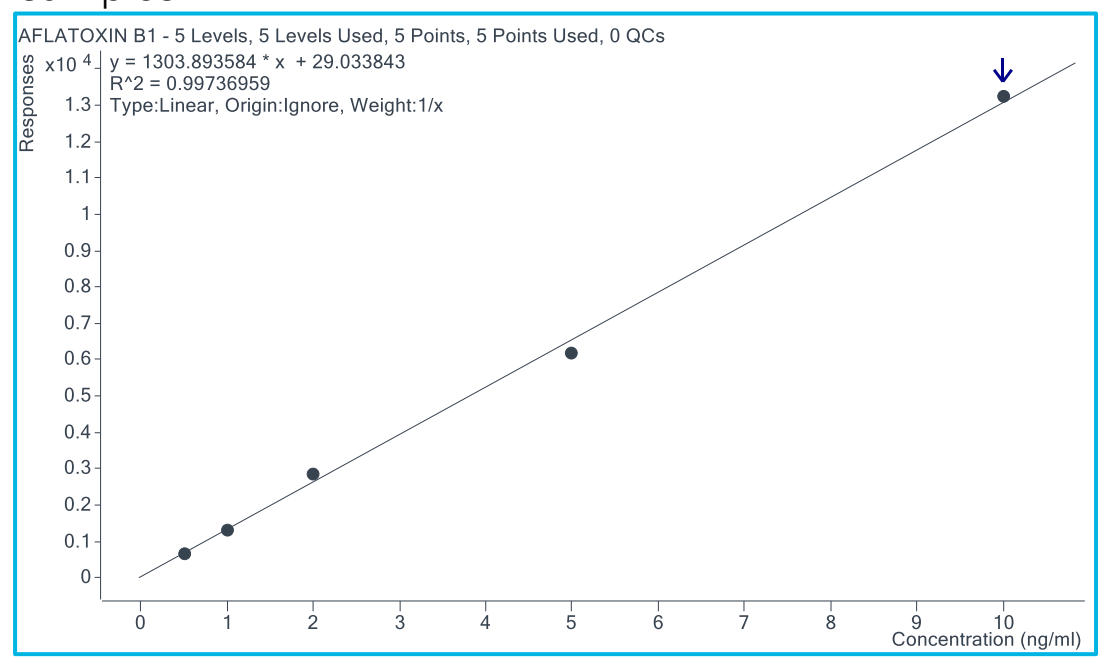

Figure 6: Procedural standard calibration curve of Aflatoxin B1

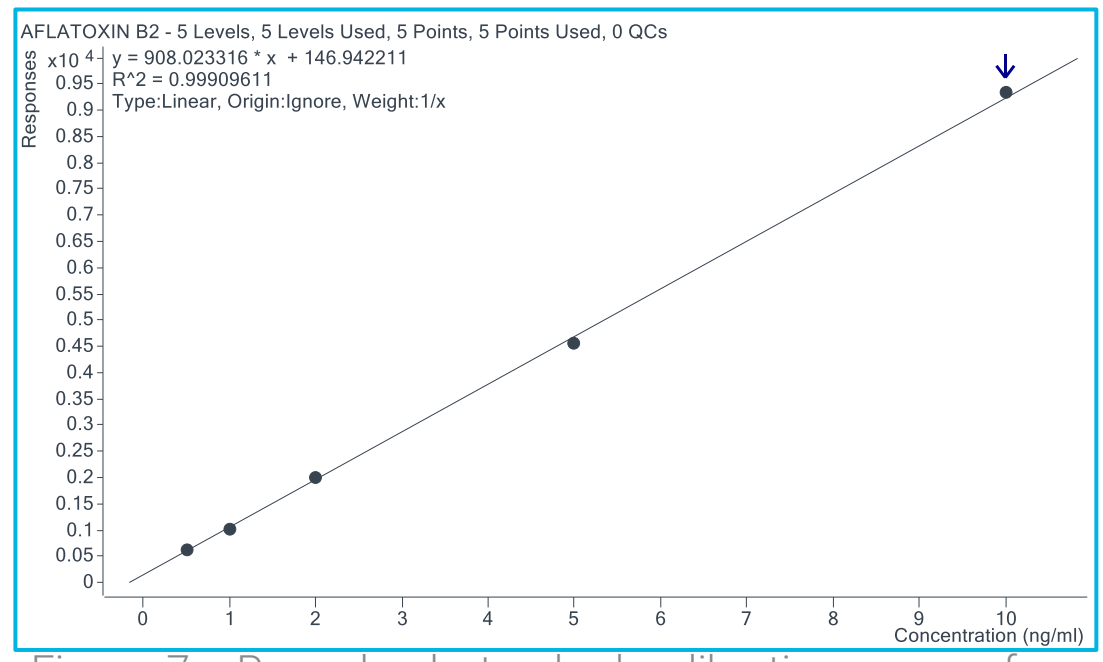

Figure 7: Procedural standard calibration curve of Aflatoxin B2

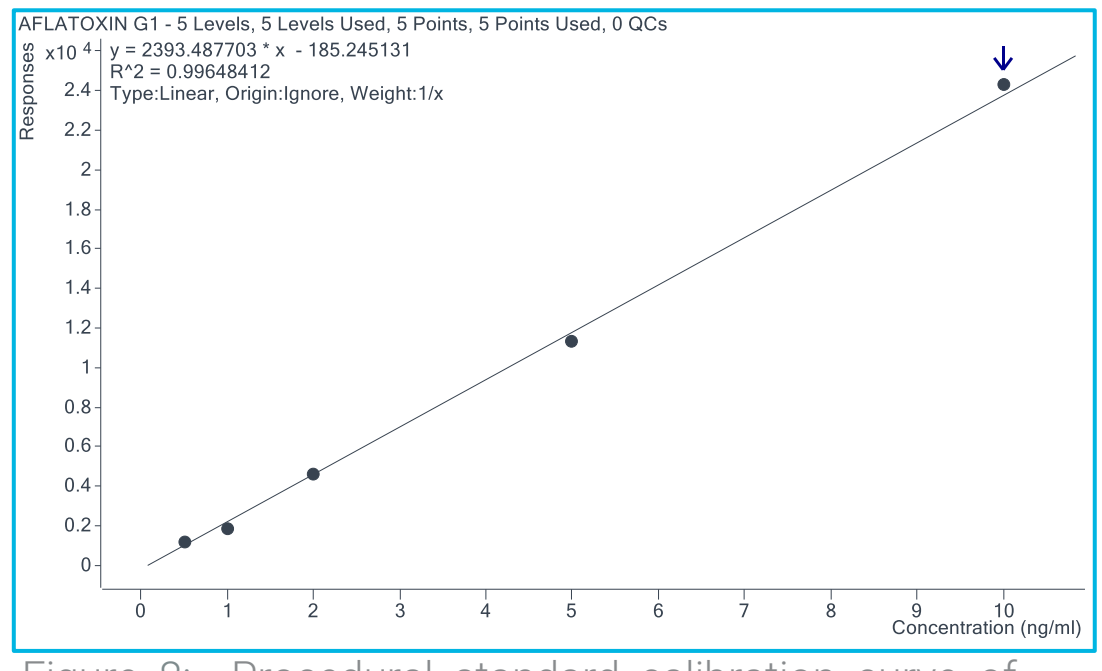

Figure 8: Procedural standard calibration curve of Aflatoxin G1

Results and Discussion

Compensating the recovery loss by procedural standard calibration

The procedural calibration standard points and the recovery samples were prepared by the same sample preparation procedure. Recoveries samples were prepared by spiking the aflatoxin standards at 30 ppb for aflatoxin B1, B2, G1 and G2 and at 0.5 ppb for aflatoxin M1, followed by the developed sample extraction and clean up. Response of recovery samples were compared with the response of procedural calibration points plotted as a calibration curve.

By adopting this approach, matrix effect and the recovery loss during the sample preparation were compensated; where recoveries of all analytes in this study were between 80-120%. This procedure is applicable especially to certain analyte/commodity combinations, especially where isotopically labelled standards are not available or are too costly. The developed method is applicable for trace level quantification of mycotoxins from milk samples containing fat and lipids.

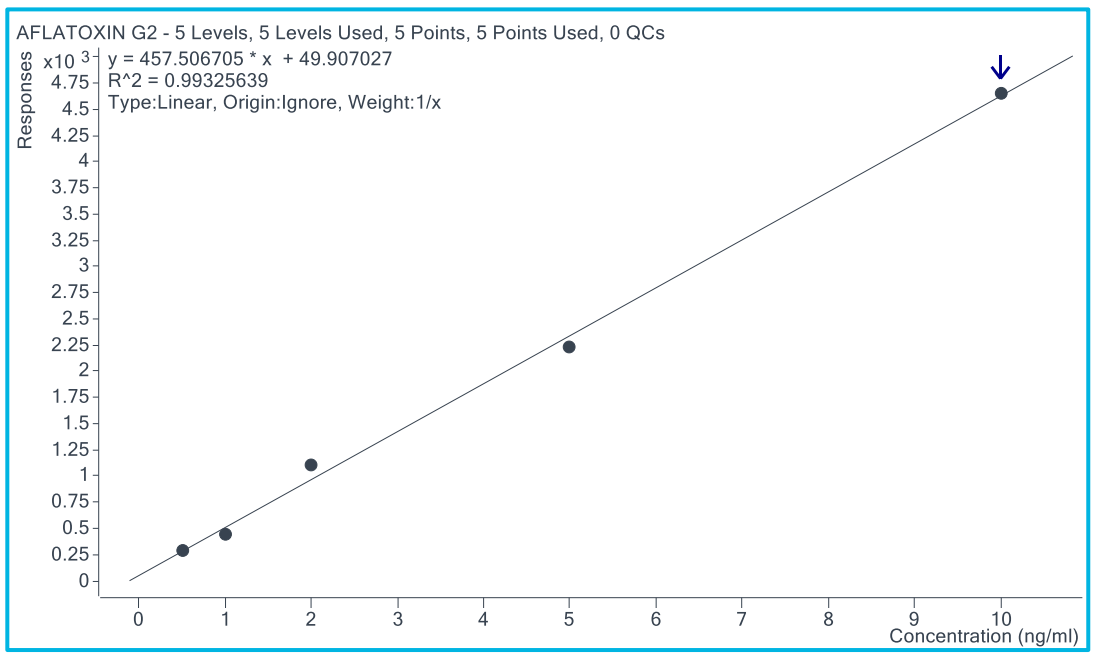

Figure 9: Procedural standard calibration curve of Aflatoxin G2

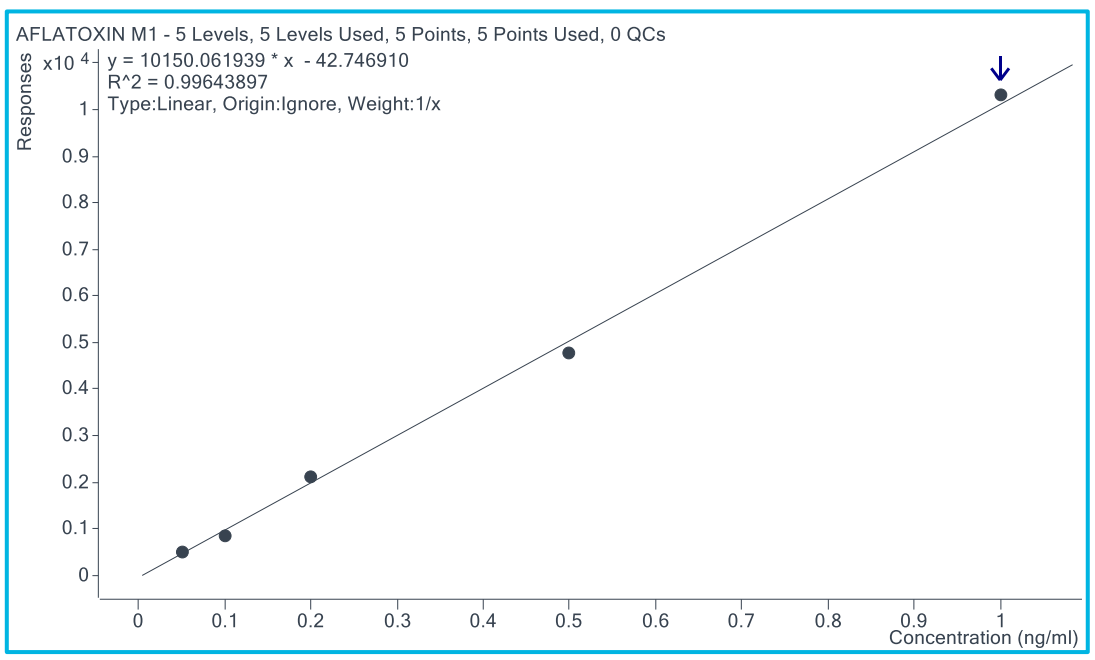

Figure 10: Procedural standard calibration curve of Aflatoxin M1

Recovery % of aflatoxins

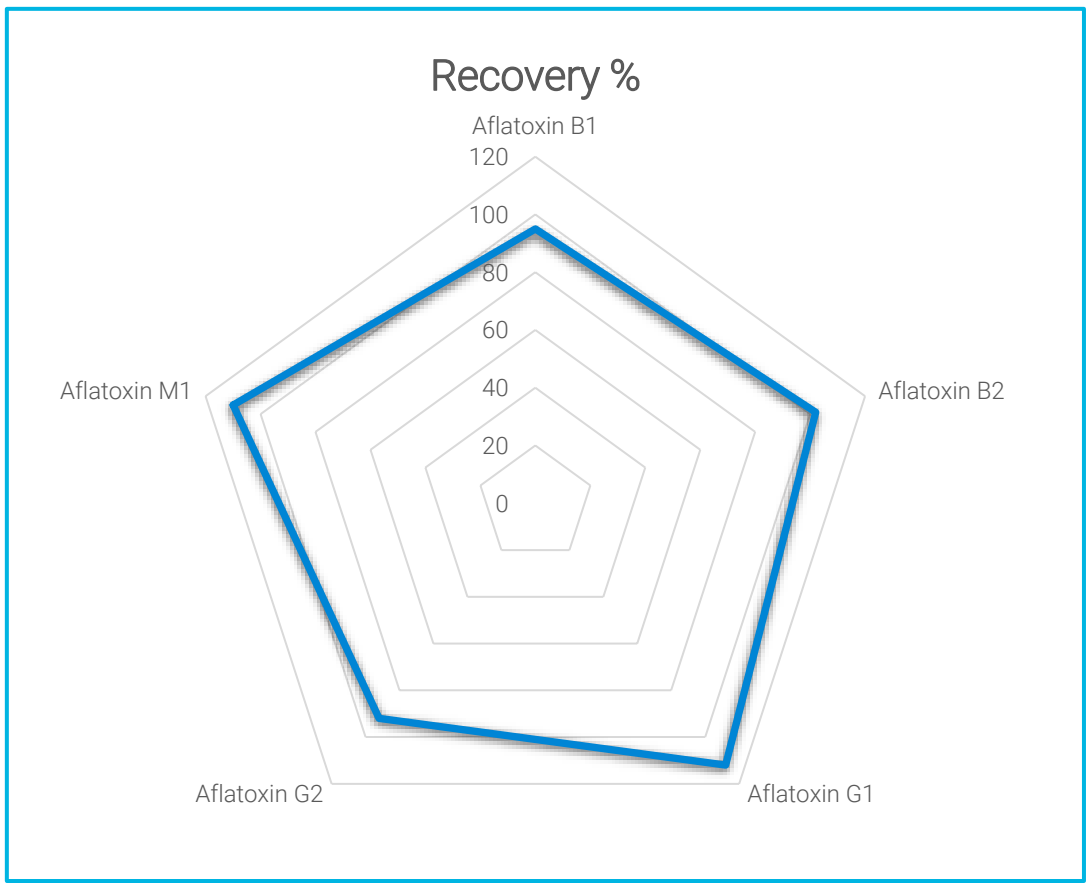

Figure 11: RADAR plot showing the recovery % of various aflatoxins in milk samples.

Conclusions

- MRM based LC-MS/MS method is developed for Aflatoxin B1, B2, G1, G2 and M1.
- Recovery % of all five analytes are calculated from the corresponding procedural standard calibration curves.
- The developed workflow demonstrated good recoveries for five mycotoxins in milk at low concentration without the use of internal standard.

References

[1]References: Iqbal S.Z. et al; Aflatoxin M1in milk and dairy products, occurrence and recent challenges: A review, Trends in Food Science and Technology 46 (2015) 110-119.

[2]Aflatoxin Analysis in Infant Formula with Enhanced Matrix Removal— Lipid by LC/MS/MS, Agilent application note 5991-6818EN

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