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A High Sensitivity LC/MS/MS Method with QuEChERS Sample Pre-treatment for Analysis of Aflatoxins in Milk Powder Samples

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Introduction

Aflatoxins (B1, B2, G1 and G2) are metabolites produced by fungi (Aspergillus favus and Aspergillus parasiticus) in crops, animal feed and dairy products. Aflatoxins are highly toxic contaminants in food and feed and their amounts increase under bad storage conditions favourite for fungal growth. Aflatoxin M1 is a hydroxylated metabolite of aflatoxin B1 found in milk of cow fed with a diet contaminated with aflatoxin B1[1]. Aflatoxin B1 is known the most carcinogenic among all the aflatoxins, and hence its metabolite aflatoxin M1 is given critical attention. Strict regulations for aflatoxin M1 in milk and dairy products have been set. For example, European Union (EU) limits the level of aflatoxin M1 to no more than 0.05 µg/kg in milk and dairy products and 0.025 µg/kg in infant food. We present a high sensitivity LC/MS/MS method for quantitative analysis of the five aflatoxins (B1, B2, G2, G2 and M1) in milk powder incorporating QuEChERS sample pre-treatment procedure, which is more cost effective as compared to the traditional procedure using immunoaffinity column (IAC)[2]. High sensitivity and good recoveries were achieved using this LC/MS/MS method.

Experimental

A mixed standard of aflatoxin B1, B2, G1 and G2 was obtained from Supelco. Aflatoxin M1 was obtained from Romer Labs. A stock solution of the mixture of 5 aflatoxins was prepared using methanol as the diluent, from which calibrant series and spiked samples were prepared. The QuEChERS kits were purchased from RESTEK. Two grams of milk powder was first extracted with the extraction kits followed by cleaning up using dSPE tubes. A LCMS-8060 triple quadrupole LC/MS/MS (Shimadzu Corporation, Japan) was used in this work. A C18 column (Kinetex, 2.1 x 100mm, 1.7um) was used for fast separation of aflatoxins using a gradient elution program. Method performance evaluation were carried out using spiked aflatoxins in milk powder samples. Table 1 shows the analytical conditions on LCMS-8060.

Shimadzu LCMS-8060, an UFMS triple quadrupole system with a heated ESI interface

Table 1: LC/MS/MS analytical conditions of aflatoxins on LCMS-8060

Column	: Kinetex C18 (2.1mmI.D x 100mmL., 1.7 µm)
Flow rate	: 0.5 mL/min
Mobile phase	: A: 5 mM ammonium acetate in water with 0.1% FA B: 5 mM ammonium acetate in MeOH
Oven temp.	: 40°C
Injection vol.	: 5 µL
Elution mode	: Gradent elution, B%: 5% (0-5 min) 50% (4- 5.5 min) 85% (6-7.5 min) 5% (8.1-10 min)
Interface	: ESI (Heated)
MS mode	: Positive, MRM, 2 transitions each compound
Interface temp.	: 350°C
Block temp.	: 400°C
DL temp.	: 250°C
CID gas	: Ar (350 kPa)
Nebulizing gas flow	: 3.0 L/min
Drying gas flow	: 10.0 L/min
Heating gas flow	: 10.0 L/min

Results and Discussion

QuEChERS sample pre-treatment

Hexane was used in the procedure to remove fats, oils and non-polar components from the milk powder samples. The extraction step was completed using Q-sep QuEChERS extraction salt packet (4 g MgSO4, 1 g NaCl, 1 g trisodium citrate dehydrate, 0.5 g disodium hydrogen citrate). Dispersive SPE tube containing MgSO4, PSA and C18 was used in the clean-up process to remove remaining water, organic acid and non-polar components respectively. The process of the sample preparation is illustrated in Figure 1.

Method Development

Automated MRM optimisation of the five aflatoxins was carried out using the LabSolutions workstation. Two MRM transitions for every aflatoxin were chosen as quantifier and confirmation ion (Table 2).

A milk powder matrix free from aflatoxins was used as a "blank" and matrix for the preparation of post-spiked calibrants to build calibration curves. The blank and every post-spiked calibrant was injected thrice and the average area was calculated to obtain reliable results.

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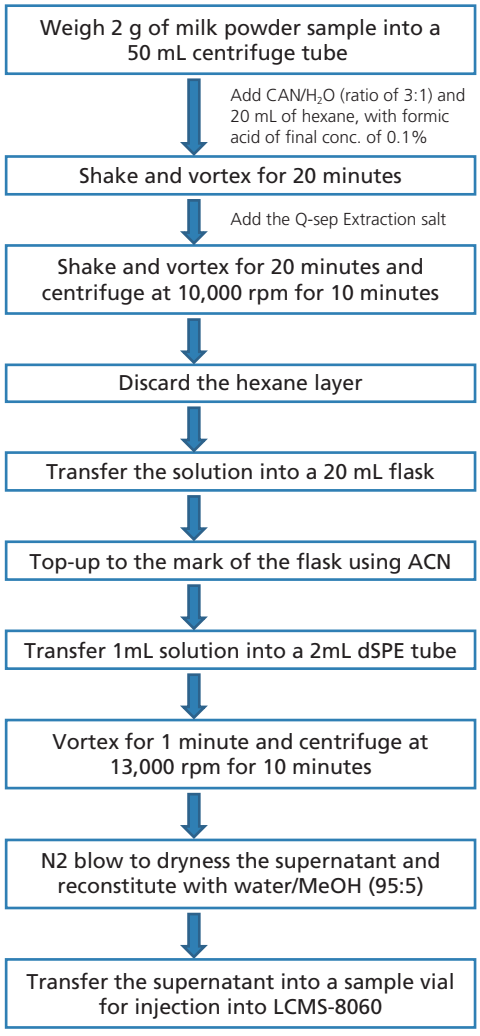

Figure 1: Flowchart of sample pre-treatment for aflatoxins in milk powders by modified QuEChERS method.

Table 2: LC/MS/MS analytical conditions of LCMS-8050 for aflatoxins

Compound	MRM (m/z)	CID Voltage (V)		
		Q1	CE	Q3
Aflatoxin B1	313.1>241.0*	-12	-40	-17
	313.1>213.0	-21	-44	-15
Aflatoxin B2	315.1>287.0*	-22	-27	-20
	315.1>259.1	-11	-30	-18
Aflatoxin G1	329.1>243.0*	-12	-28	-17
	329.1>200.0	-12	-40	-22
Aflatoxin G2	331.1>189.0*	-24	-43	-19
	331.1>245.0	-12	-31	-18
Aflatoxin M1	329.0>273.0*	-12	-23	-18
	329.0>259.0	-23	-24	-29

* MRM transitions used as quantifiers.

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A chromatogram of spiked sample is shown in Figure 2. Linear calibration curves were obtained for all five aflatoxin compounds with good linearity (r^2 >0.999). The calibration curves of aflatoxins spiked in milk powder matrix are shown in Figure 4.

Method Performance Evaluation

The LOD and LOQ of aflatoxins in milk powder matrix are lower than 0.83 pg/mL and 2.50 pg/mL respectively (Table 3). The repeatability of the method was evaluated using spiked samples at two concentrations. The peak area %RSD of aflatoxins were found to be lower than 7.46%.

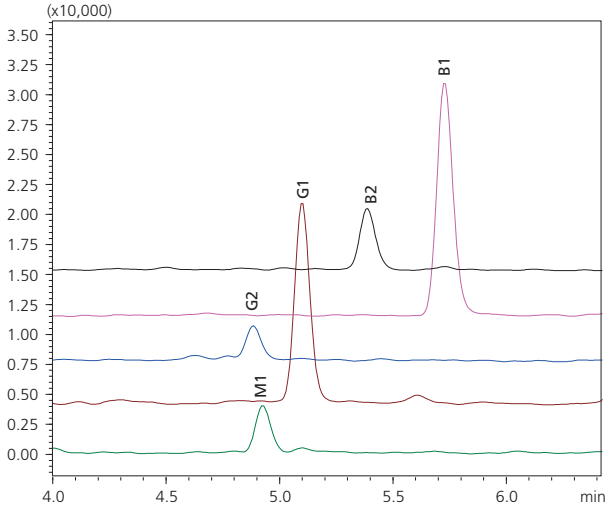

Figure 2: Total ion chromatogram of 5 aflatoxins (Concentrations of B1,G1 and M1 at 10 pg/mL; B2 and G2 at 3 pg/mL)

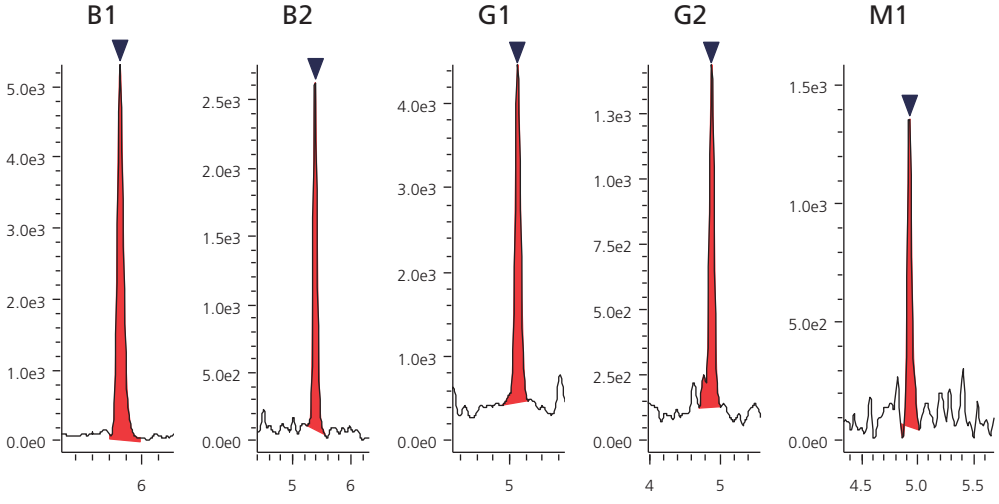

Figure 3: Single LOQ MRM chromatograms of 5 aflatoxins
(Concentrations of B1, G1 and M1 (5 pg/mL); B2 and G2 (3 pg/mL)

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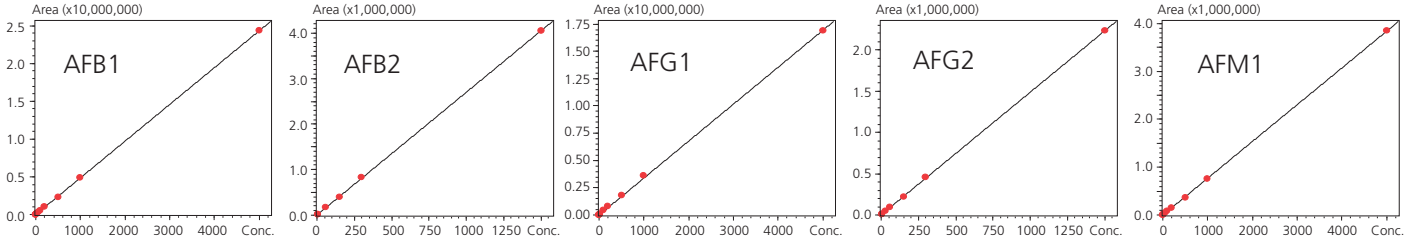

Figure 4: Calibration curves of aflatoxins B1, B2, G1, G2 and M1 in milk powder matrix.

Table 3: LOD, LOQ and repeatability of aflatoxin spiked samples at different concentrations

Aflatoxin	Range (pg/mL)	Linearity	LOD (pg/mL)	LOQ (pg/mL)	%RSD (n=6)			
					5 pg/mL	6 pg/mL	30 pg/mL	50 pg/mL
B1	1-5000	0.9999	0.14	0.44	3.1			2.3
B2	3-1500	0.9999	0.36	1.09		6.4	2.4	
G1	3-5000	0.9998	0.71	2.16	4.0			2.44
G2	3-1500	0.9999	0.41	1.22		5.8	3.5	
M1	3-5000	0.9999	0.83	2.50	7.5			2.7

Both the matrix effect and recoveries of aflatoxins were evaluated by using a duplicate set of samples at different concentrations. Each duplicate was obtained from the average of three injections. The results are shown in Table 4 and Table 5.

Table 4: Matrix effects of the MRM method for aflatoxins in spiked milk powder samples

Concentration (pg/mL)	Matrix effect (%)			Concentration (pg/mL)	Matrix effect (%)	
	B1	G1	M1		B2	G2
5.0	105.1	116.0	99.4	6.0	105.8	116.3
50.0	105.3	107.9	105.4	30.0	110.2	109.3

Table 5: Recoveries of aflatoxins in spiked milk powder samples

Concentration (pg/mL)	Recovery (%)			Concentration (pg/mL)	Recovery (%)	
	B1	G1	M1		B2	G2
5.0	76.6	87.3	83.8	6.0	71.6	70.8
50.0	73.8	76.5	75.6	30.0	73.9	75.6

Analysis of aflatoxins in actual milk powder samples

Three milk powder samples from local supermarket were analysed using the established MRM method. The results showed that no aflatoxin was detected in all three samples.

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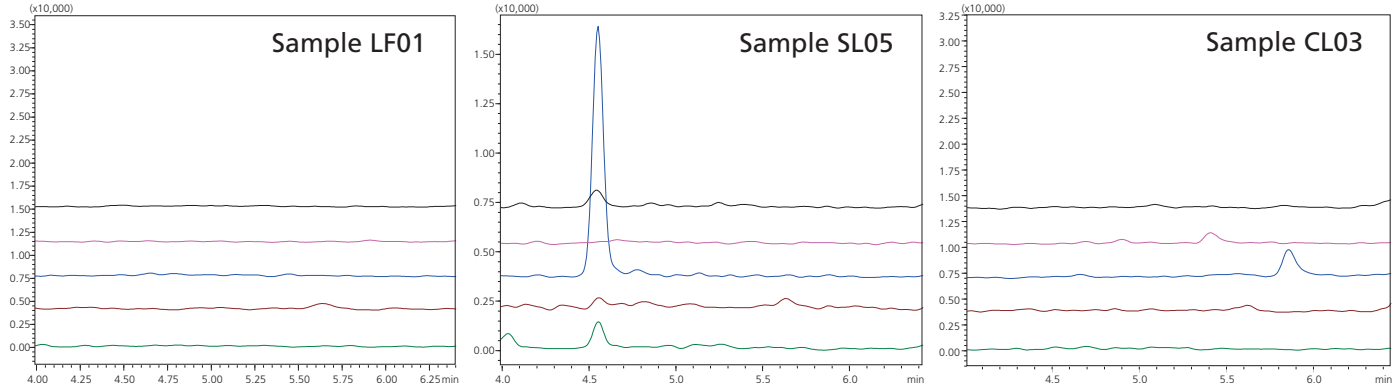

Figure 5: MRM Chromatograms for Aflatoxin B2, B1, G2, G1 and M1 (top to bottom) of three milk powder samples from local supermarket. Targets were not detected in all samples.

Conclusions

A high sensitivity LC/MS/MS method with QuEChERS for sample pre-treatment was established using Shimadzu LCMS-8060 system. The QuEChERS sample preparation method was proven effective and easy to operate. The method performance including sensitivity, linearity, repeatability, matrix effect and recovery were carried out and the results confirm that the method is feasible and reliable for determination of aflatoxins in milk powder samples.

References

1. Iqbal, S. Z.; Jinap, S.; Pirouz, A. A.; Ahmad Faizal, A.R. *Trends in Food Science & Technology* 2015, *46*, 110-119.
2. Cherkani-Hassani, A.; Mojemmi, B.; Mouane, N. *Trends in Food Science & Technology* 2016, *50*, 56-69.

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