PO-CON1739E

SHIMADZU
Excellence in Science

Solid Phase Extraction and SFC-MS/MS Method for Analysis of Aflatoxins M1, M2, B1, B2, G1 and G2 in Milk Powders

ASMS 2017 TP 210

Jun Xiang Lee[1], Yin Ling Chew[1], Jie Xing[1] and Zhaoqi Zhan[1]
1. Application Development & Support Centre, Shimadzu (Asia Pacific) Pte Ltd, 79 Science Park Drive, Singapore

Solid Phase Extraction and SFC-MS/MS Method for Analysis of Aflatoxins M1, M2, B1, B2, G1 and G2 in Milk Powders

Introduction

Aflatoxins B1, B2, G1 and G2 are secondary metabolites that are produced by Aspergillus fungi. If aflatoxins B1 and B2 are present in feeds consumed by lactating animals, they can be converted to hydroxylated metabolite aflatoxins M1 and M2 in the animals respectively, which are found eventually in the milk [1]. Aflatoxins B1, B2, G1, G2 are carcinogenic and aflatoxin M1 is potentially carcinogenic. The levels of these aflatoxins in dairy products like milk powders are strictly regulated around the world. For example, European Union regulation limits aflatoxin M1 in milk below 0.050 µg/kg [2]. Many LC/MS/MS methods were reported for analysis of aflatoxins B1, B2, G1, G2 and M1, but few including aflatoxin M2 [4]. In addition, sample preparation method is varied for different matrixes and is often a critical factor to the analysis sensitivity and accuracy. The aim of this study is to develop a novel method - supercritical fluid chromatography SFC-MS/MS for high sensitivity analysis of aflatoxins B1, B2, G1, G2, M1 and M2 in milk powders. The supercritical fluid (SF) CO2 mobile phase has advantages of cost-effective and environmental friendly as compared to organic solvents. A special sample preparation procedure was explored, which involved extraction with adding Q-Sep extract salt and purification with Supel™ Tox Alfazea SPE cartridge. Conventional SPE uses the concept of binding target compounds to the stationary phase first and eluted out by organic eluent subsequently. However, Supel™ Tox Alfazea SPE cartridge acts as a filter where the sample matrix is trapped while the target aflatoxins are eluted out.

Experimental

Materials and analytical conditions

Aflatoxins standards (B1, B2, G1, G2, M1 and M2) were obtained from Supelco and Romer Labs. Mixed standards of calibration series and spiked samples were prepared in pure acetonitrile. The SPE cartridges (Supel™ Tox Alfazea) were obtained from Supelco and extraction salts (Q-Sep) were obtained from Restek. A Shimadzu LCMS-8050 LC/MS/MS coupled with Nexera UC, a supercritical fluid chromatography (SFC) system, was employed to develop a MRM method for quantitative analysis of the aflatoxins. A Shim-pack UC-X Sil (250 mmL x 2.1mm I.D., 3µm) column was adopted and a gradient elution program was set up for separation of the six compounds. The detailed conditions are compiled into Table 1.

Solid Phase Extraction and SFC-MS/MS Method for Analysis of Aflatoxins M1, M2, B1, B2, G1 and G2 in Milk Powders

Table 1: Analytical conditions for six aflatoxins on SFC-MS/MS with LCMS-8050

Column	: Shim-pack UC-X Sil (250 mmL. x 2.1mm I.D., 3µm)
Flow Rate	: 1.0 mL/min
	0.4 mL/min (make up pump)
Mobile Phase	: A : Carbon dioxide (supercritical fluid)
	B : Methanol with 5 mM ammonium formate
	C : Methanol with 0.1% formic acid
Oven Temp.	: 40 °C
Injection vol.	: 5 µL
Elution Mode	: Gradient elution, LC program 7min
	10% B (0.00 mins to 2.00 mins) → 30% B (2.50 mins to 3.50 mins)
	→ 10% B (3.60 mins to 5.00 mins)
Interface	: ESI
MS Mode	: MRM, Positive
Block Temp.	: 400 °C
DL Temp.	: 250 °C
Interface Temp.	: 350 °C
CID gas	: Ar (350 kPa)
Nebulizing Gas Flow	: Nitrogen, 1.5 L/min
Drying Gas Flow	: Nitrogen, 5.0 L/min
Heating Gas Flow	: Zero air, 15 L/min

Sample preparation

Five grams of milk powders were extracted with 20 mL of mixed solvent (water and acetonitrile in 1:1) with 0.1% formic acid. The extraction salts pack was added and vortexed for 20 mins to facilitate the extraction of analytes efficiently. The sample was then centrifuged at 11,000 rpm for 10 mins and the supernatant was transferred for SPE. The Supel™ Tox Alfazea SPE tubes were set up on a vacuum manifold and conditioned with water and acetonitrile before sample loading and washing with acetonitrile. The clear solution obtained was filtered with 0.22 µm PTFE filter before injection into the LCMS-8050 system.

Results and Discussions

Development of SFC-MS/MS method for six aflatoxins

To facilitate ionisation, an additional make-up solvent MeOH with 0.1% formic acid is used. Automated MRM optimisation was performed under the SFC conditions to obtain two transitions ions for each aflatoxin, one as quantifier and the other as confirmation ion. In general, the transition with higher intensity is often chosen as the quantifier ion. However, due to more interferences lead to high baselines, the quantifier MRM for B1, B2 and G1 selected in this study are the transition ions with lower intensity but yielding better S/N ratio (Table 2).

A gradient elution SFC program was set up for fast separation of the six aflatoxins in 7 minutes. The high permeability of the SF-CO2 allows a higher mobile phase flow rate (1.4 mL/min) without high back-pressure. The gradient program starts with a high percentage of SF-CO2 at 90% for 2 minutes. This step is to assist in eluting non-polar interferences from milk powder extract. The percentage of modifier solvent (MeOH with 0.1% FA) is then increased to 30% (Table 1). This increases the eluting strength of the mobile phase which elutes the aflatoxin compounds.

The elution principle of SFC can be likened to a normal phase LC process. Less-polar compounds will elute first followed by more polar compounds. In this study, aflatoxin B1 and B2 are the least polar compounds while their hydroxylated forms M1 and M2 are the most polar one. As expected, aflatoxin B1 and B2 elute first, while M1 and M2 elute at the last (Figure 1).

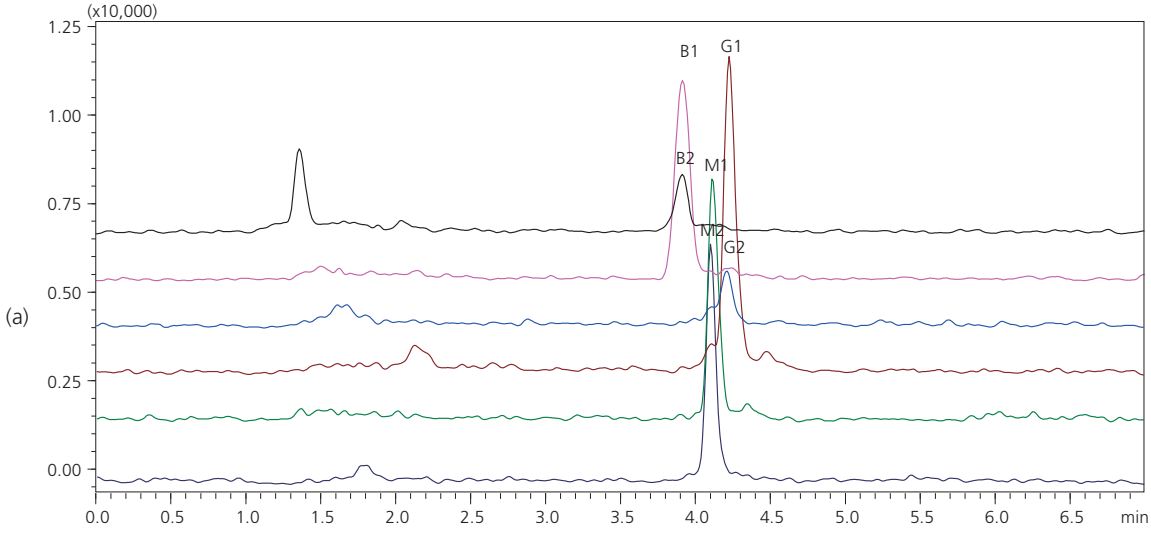

Figure 1: MRM chromatograms of aflatoxins mixed standards B1, G1, M1 and M2 at 100pg/g; B2 and G2 at 30 pg/g.

Solid Phase Extraction and SFC-MS/MS Method for Analysis of Aflatoxins M1, M2, B1, B2, G1 and G2 in Milk Powders

Establishment of MRM quantitation method

Each post-spiked calibrants was injected thrice to obtain an average peak area for calibration curve construction. Calibration curves with good linearity (r2>0.999) were obtained for all six aflatoxins for concentration up to 1200 or 4000 ng/kg (Figure 2). The SFC-MS/MS method exhibited low limit of detection (LOD) ranging from 7.6 ~ 22.4 ng/kg and low limit of quantitation (LOQ) ranging from 22.8 ~ 68.0 ng/kg. The repeatability of the method was evaluated on spiked samples at 100 ng/kg , 400 ng/kg and 1000 ng/kg (B1, G1, M1 and M2) and 120 ng/kg and 300 ng/kg (B2 and G2). Good repeatability was obtained for the spiked sample with %RSD results (n=6) ranging from 2.3 ~ 14.6%. The linearity, LOD, LOQ and %RSD results are summarised in Table 2.

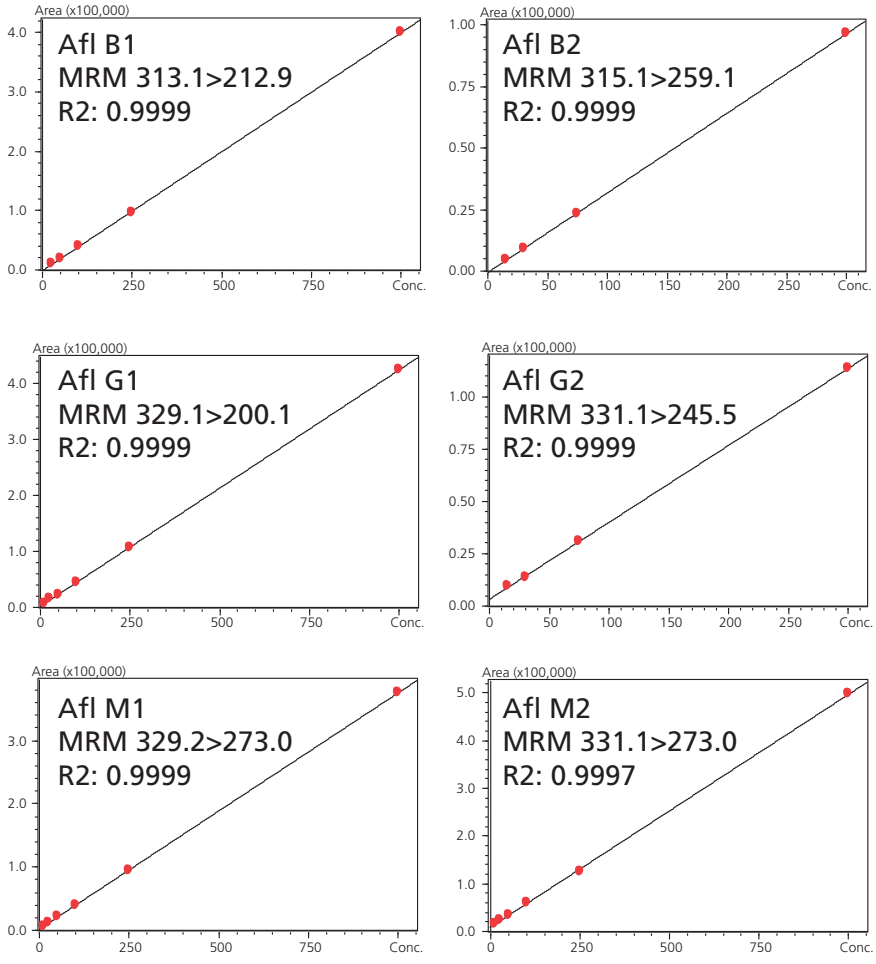

Figure 2: Calibration curves of aflatoxin B1, B2, G1, G2, M1 and M2 in spiked samples.

Solid Phase Extraction and SFC-MS/MS Method for Analysis of Aflatoxins M1, M2, B1, B2, G1 and G2 in Milk Powders

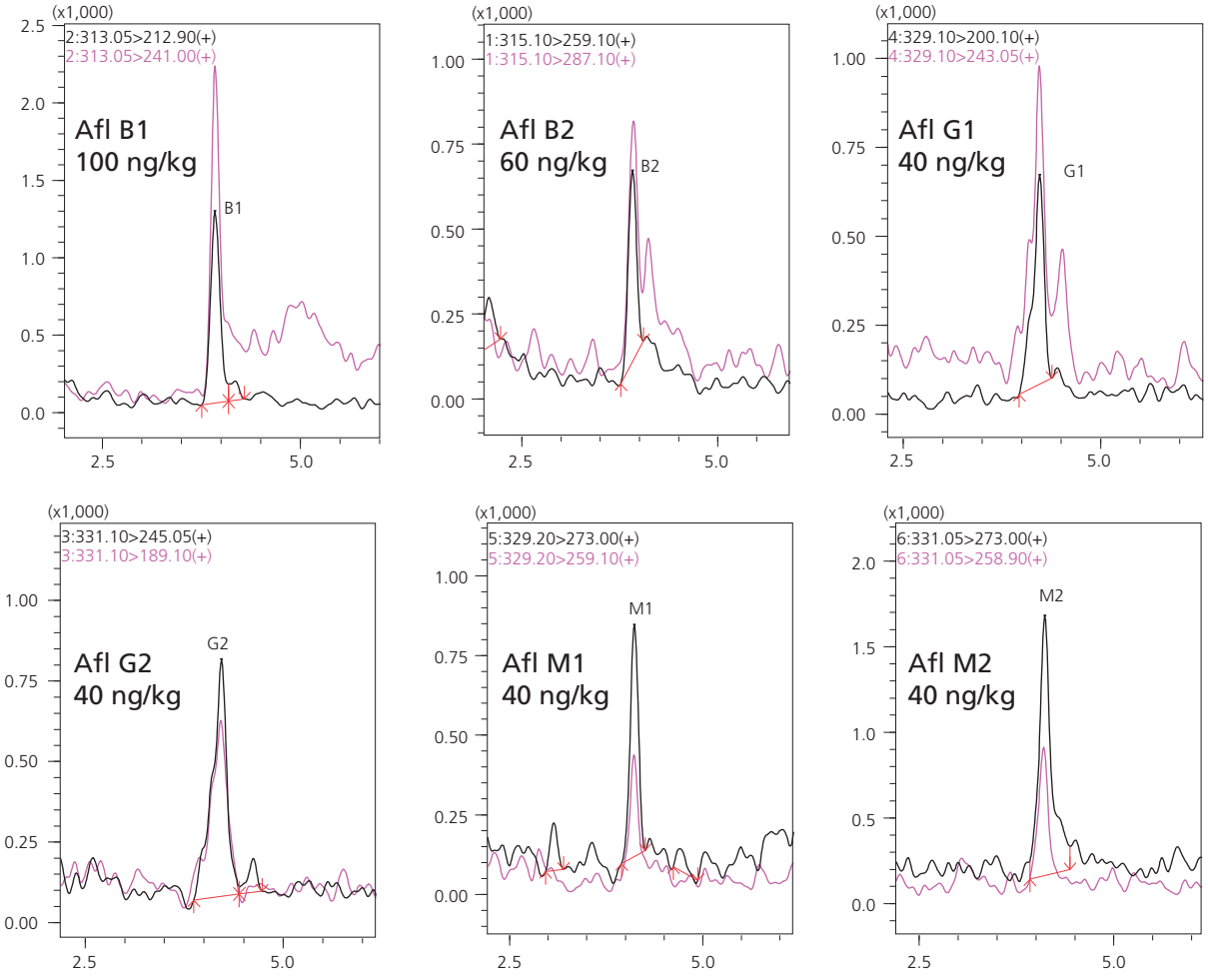

Figure 3: Individual MRM peaks of B1 at 100 ng/kg; G1, M1 and M2 at 40 ng/kg; B2 and G2 at 60 ng/kg in spiked samples

Table 2: Calibration curves and performance of SFC-MS/MS method for quantitative determination of six aflatoxins in milk powders on LCMS-8050

No	Aflatoxin	MRM Transition	RT (min)	Linearity range (ng/kg)	R2	LOD range (ng/kg)	LOQ range (ng/kg)	%RSD (n=6)		
								25 (ng/kg)	100 (ng/kg)	250 (ng/kg)
1	B1	313.1>212.9	4.02	100~4000	0.9999	22.4	68.0	6.5	2.3	2.9
2	B2	315.1>259.1	3.93	60~1200	0.9999	19.2	58.4	N.A.	8.2	6.0
3	G1	329.1>200.1	4.30	40~4000	0.9999	7.6	22.8	12.6	5.5	4.9
4	G2	331.1>245.1	4.18	60~1200	0.9998	15.2	46.4	N.A.	3.6	1.8
5	M1	329.2>273.0	4.18	40~4000	0.9999	10.0	30.0	14.6	6.6	4.6
6	M2	331.1>273.0	4.14	40~4000	0.9997	11.2	34.4	10.4	4.4	6.1

Solid Phase Extraction and SFC-MS/MS Method for Analysis of Aflatoxins M1, M2, B1, B2, G1 and G2 in Milk Powders

Matrix effect and recovery of SFC-MS/MS method

Sample preparation is a critical factor to obtain good recovery and less matrix effect of the aflatoxin analysis method in milk powders. The matrix effect and recoveries were evaluated with spiked milk powder samples at 3 concentration levels L1, L2 and M. The L1 level corresponds to 100 ng/kg for B1, G1, M1 and M2; 30 ng/kg for B2 and G2. The concentrations of L2 level are four times higher than that of L1, 400 ng/kg for B1, G1, M1, M2 and 120 ng/kg for B2 and G2. The M level spiked samples were prepared from L1 level samples by adding an evaporation step after the SPE. The obtained acetonitrile solution was concentrated one time by N2 blowing at room temperature to obtain final concentrations of 200 ng/kg for B1, G1, M1, M2 and 60 ng/kg for B2, G2.

The results compiled in Table 3 indicate that, the six aflatoxins displayed recoveries of 70.2% ~ 134.5%. For L1 and L2 samples, aflatoxins B1, B2, G1 and G2 displayed negligible matrix effect of 81.8%~129.2% while aflatoxins M1 and M2 displayed a stronger matrix effect of 136.4~173.9%. In general, aflatoxin M1 and M2 exhibits relatively lower recovery and higher matrix effect.

Table 3: Recovery and matrix effect of six aflatoxins in milk powder samples (n=3)

Aflatoxin	Recovery (%)			Matrix effect (%)		
	L1	L2	M	L1	L2	M
B1	101.4	112.8	96.7	87.3	81.8	25.1
B2	ND	127.9	124.0	ND	83.4	22.8
G1	77.5	109.8	78.7	129	91.9	31.6
G2	ND	97.5	132.7	ND	127.8	33.4
M1	73.7	70.2	110.2	156	136.4	28.9
M2	84.6	71.3	134.5	145	173.9	28.3

L1: 100 ng/kg for B1, G1, M1 and M2; 30 ng/kg for B2 and G2
L2: 400 ng/kg for B1, G1, M1 and M2; 120 ng/kg for B2 and G2
M: 200 ng/kg for B1, G1, M1 and M2; 60 ng/kg for B2 and G2; (2x concentration)

Furthermore, the M level sample exhibits significant matrix effect (23% ~ 33%) due to the additional concentration step after the SPE using Supel™ Tox Alfazea cartridge. This phenomenon indicates that interferences are present in the final purified solutions.

Solid Phase Extraction and SFC-MS/MS Method for Analysis of Aflatoxins M1, M2, B1, B2, G1 and G2 in Milk Powders

Conclusions

A SFC-MS/MS analytical method is developed for quantitative analysis of aflatoxins M1, M2, B1, B2, G1 and G2 in milk powders. A special sample preparation procedure is adopted, which includes extraction with adding of Q-Sep extract salt and purification with Supel™ Tox Alfazea SPE cartridge. This new procedure was proven to be efficient in extraction of the six aflatoxins in milk powders and in the subsequent purification for high sensitivity MRM based analysis method. The LOQs of the method for the six aflatoxins is at 22.8~68.0 ng/kg for aflatoxin B1, B2, G1 and G2, and are 30.0 ng/kg and 34.4 ng/kg for aflatoxin M1 and M2, respectively.

References

1. Benkerroum, N. International Dairy Journal (2016), 62, 63–75.
2. Commission Regulation (EC) No 1881/2006, http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02006R1881-20140701&from=EN
3 Sartori, A. C. A. V.; Mattos, J. S. de; Moraes, M. H. P. de; Nóbrega, A. W. da. Food Anal. Methods (2015), 8, 2321-2330

Disclaimer: The products and applications in this poster presentation are intended for Research Use Only (RUO). Not for use in diagnostic procedures.

First Edition: June, 2017

SHIMADZU

Shimadzu Corporation

www.shimadzu.com/an/