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Development and Validation of on-line SFE-SFC-MS/MS Method for Screening of Aflatoxins B1, B2, G1 and G2 in Grain Matrices

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❑ Introduction

Aflatoxins B1, B2, G1 and G2 are produced by Aspergillus fungi, which are known to have carcinogenic effects in human. Approaches to determine these analytes in various food matrices often require the use of substantial volumes of organic solvent in manual extraction and clean-up procedure such as immunoaffinity columns [1-2]. Supercritical fluid carbon dioxide (SFCO2), an environmentally-benign solvent, has received increasing attention as an alternative in extraction and chromatography. Integrating supercritical fluid extraction (SFE) and chromatography (SFC) using SFCO2, the Nexera UC system (Shimadzu) allows fully-automated on-line sample extraction and analysis, which eliminates the tedious steps of manual operation of sample pre-treatment and reduces the running cost. This novel system was applied successfully in analysis of contaminants in agricultural products and PFCs in textiles [3-4]. We describe here a further application of the SFE-SFC-MS/MS platform in fully-automated screening from extraction to quantitative analysis of aflatoxin B1, B2, G1 and G2, which are regulated strictly in food and agricultural products [5]. Improved accuracy in quantitation was obtained by using internal standard method, which compensates largely the variation due to different matrices in SFE and matrix effect in MS analysis.

Table 1. LC and MS acquisition parameters

Column	:	COSMOSIL 5PBr (4.6 mm I.D. × 250 mm, 5.0 μm)
Mobile phase	:	A: Supercritical fluid carbon dioxide ($SFCO_2$) B: 10mM Ammonium acetate in methanol C: 0.1% Formic acid (v/v) in methanol
Flow rate of mobile phase	:	5 mL/min (0-2 min), 3 mL/min (2.01-10 min) 3 mL/min (make up pump of MS)
Column temp.	:	40 °C
Elution mode	:	**SFE program** Static: 0 to 4 min, B conc.: 20% (0-2 min), 5% (2.01-4 min) Dynamic : 4 to 10 min, B conc.: 5% (4.01-10 min) **SFC elution program** B. Conc. 25 % (10.01-20.5 min), 50% (20.51 to 21.50 min)

Interface & temp.	:	ESI, 300°C
MS mode	:	Positive, MRM
Block temp.	:	400°C
DL temp.	:	250°C
Nebulizing gas flow	:	N_2, 1.5 L/min
Drying gas flow	:	N_2, 5 L/min
Heating gas flow	:	0 air, 10 L/min

❑ Experimental

Analytical conditions and sample preparation

Aflatoxin standards (B1, B2, G1, G2) and a mixture of ^{13}C-labelled internal standards (^{13}C IS) were acquired from Supelco and Romerlabs, respectively. Mixed standards and ^{13}C IS for calibration series and spiking were prepared in methanol. For construction of calibration series, 10 µL of mixed aflatoxin standards and 10 µL of mixed IS were pre-spiked into absorbent (Miyazaki Hydro-Protect powders) before loading into the 5mL extraction vessel (stainless steel). For sample preparation, 1g of food sample was weighed and mixed thoroughly with water, acetonitrile, absorbant and 10uL of mixed internal standards (100 ng/mL) before loading into a 5mL extraction vessel.

Figure 1. Nexera UC, a SFE-SFC-LC/MS/MS system

A Shimadzu Nexera UC connected to LCMS-8050 was used for development of online extraction and quantitative analysis of Aflatoxin B1, B2, G1 and G2 in maize and peanut CRMs, and food matrices. A COSMOSIL 5PBr (250 x 4.6 mm, 5 µm) column was used for SFC analysis of the four aflatoxins. The analysis cycle includes 10 min of extraction (SFE) and 11.5 min of elution and analysis (SFC-MS/MS).

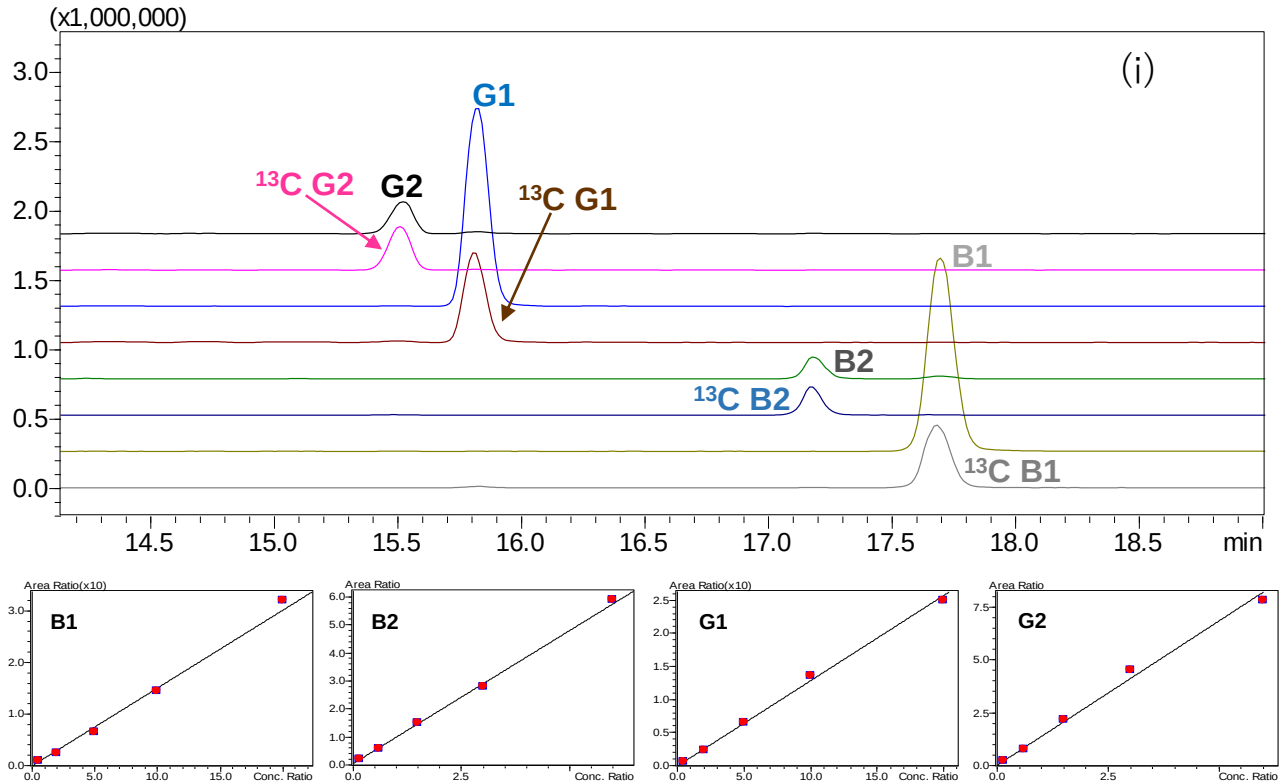

Figure 2. (i) Total MRM chromatograms of aflatoxin standards B1 and G1 (2 ng) and B2 and G2 (0.6 ng) with 13C ISs (1ng each) and (ii) IS calibration curves for B1, B2, G1 and G2 in absorbent (Miyazaki Hydro-Protect powders)

❑ Results and Discussion

Development and validation of on-line SFE-SFC/MS/MS method

An online SFE-SFC/MS/MS method was established for 4 aflatoxins in peanut flour and cereal samples. Two MRM transitions (quantifier and confirmation ions) are required for peak detection. A SFE programme was set up for the extraction of the 4 aflatoxins. Splitting of flowline was applied in the extraction method to reduce the amount of matrix interference entering into the system, in order to prevent contamination. Internal standards were also used to correct for the matrix effect and recovery due to different matrix composition. A SFC gradient elution method was set up for the separation of the 4 aflatoxins. Besides using SFCO2 as the mobile phase, a modifier solvent, 10 mM ammonium acetate in methanol was used to increase the solvent strength for optimization of separation of aflatoxins. A make-up solvent, 0.1% formic acid in methanol, was added after-column to enhance the ESI ionisation of the compounds for MS detection.

A quantitation method was set up on the SFE-SFC-MS/MS system, with a calibration range of 0.15~6 ng for aflatoxins B2 and G2 and 0.5~20 ng for aflatoxins B1 and G1. Calibration series were fabricated based on peak area and absolute amount (ng) ratio of aflatoxins to that of ^{13}C IS. Linear calibration curves with R^2 >0.991 was consistently obtained over a period of three days. (**Table 2**)

Method accuracy was estimated by repeated analysis of maize and peanut flour CRMs obtained from FAPAS. Satisfactory results were obtained for both types of reference materials, with average recoveries of 87.5% to 134% of the actual assigned value. Good repeatability was obtained with RSD < 15% (n=6) across all three days. (**Table 3**)

Table 2. Calibration curves and linearity of aflatoxins for method validation across 3-days inter-day experiment.

No	Aflatoxin	RT (min)	Quantifier transition	Reference Transition	Range (ng)	Linearity
1	B1	17.58	312.90>285.05	312.90>241.10	0.5 - 20	0.991 ~ 0.998
2	13C AFB1	17.56	330.00>300.95	330.00>255.10		
3	B2	17.09	315.10>286.85	315.10>242.90	0.15 - 6	0.991 ~ 0.998
4	13C AFB2	17.08	331.90>303.10	331.90>257.10		
5	G1	15.75	328.80>243.15	328.80>200.05	0.5 - 20	0.994 ~ 0.998
6	13C AFG1	15.74	345.90>257.20	345.90>212.15		
7	G2	15.46	331.10>245.10	331.10>189.00	0.15 - 6	0.991 ~ 0.999
8	13C AFG2	15.45	348.10>301.05	348.10>259.15		

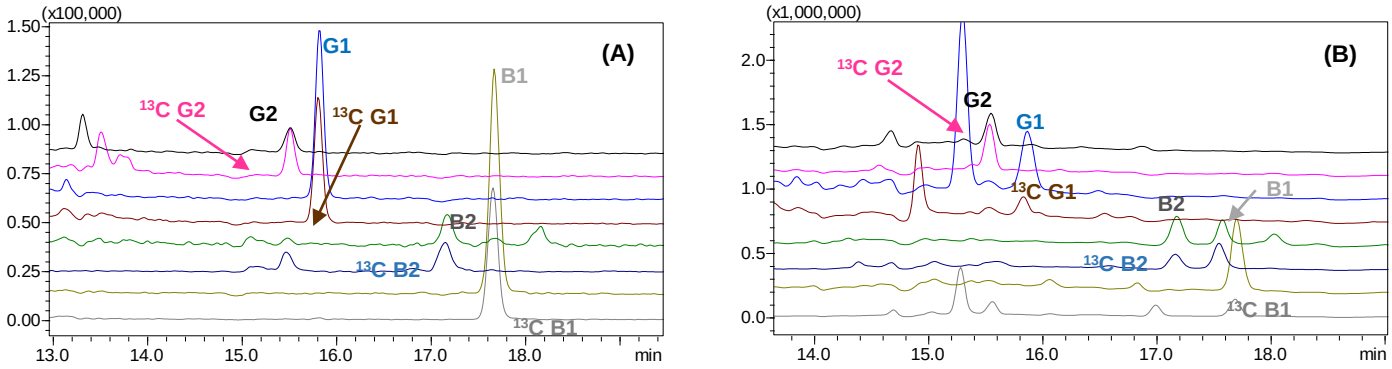

Figure 3. Total MRM chromatograms of aflatoxins with 13C ISs (1ng each) in maize CRM (A), peanut CRM (B)

Table 3. Quantitation results of aflatoxins in maize and peanut CRM samples for method validation.

CRM		Assigned value (ng)*		Accepted range (ng)*	Conc. (ng)	Accuracy (%)	Inter day RSD (%)	LOD (ng/g)
MAIZE	1	B1	3.80	2.13 – 5.47	3.59	94.4	4.5	0.05
	2	B2	1.89	1.06 – 2.72	2.53	134.0	3.6	0.05
	3	G1	1.07	0.60 – 1.54	1.11	104.0	2.3	0.05
	4	G2	1.00	0.56 – 1.44	1.00	99.7	9.3	0.05
	Total		7.67	4.30 – 11.05	8.23	110.2	NA	
PEANUT	5	B1	3.42	1.93 – 4.92	2.99	87.5	10.5	0.2
	6	B2	1.88	1.05 – 2.70	1.88	99.8	14.9	0.15
	7	G1	1.77	0.99 2.54	2.12	119.8	14.7	0.15
	8	G2	0.96	0.54 – 1.38	0.91	95.1	13.9	0.15
	Total		8.29	4.64 – 11.94	7.90	95.3	NA	

*Note: Except for LOD, all results were summarized from six repeated injection (n=6) across 3 days inter-day experiment. *values set by FAPAS.*

Detection limit of aflatoxins in maize and peanut flour

Detection limit was estimated based on firm detection of aflatoxins at the lowest spiking amount from 6 repeated injections. In addition to peak confirmation with two MRM transitions for each compound, detection criteria also includes the signal-to-noise ratio (S/N) of greater than 3. It was noted that different matrices have different detection limits due to the varying matrix interferences present. For maize samples, the detection limit achieved by the SFE-SFC-MS/MS method was comparable with that achieved using conventional HPLC-RF method with sample clean-up using immunoaffinity column, while the detection limit in peanut matrix was higher (**Table 3**). Nevertheless, the detection limits achieved in both types of food matrices are fit for the purpose of regulatory compliance testing in Singapore.

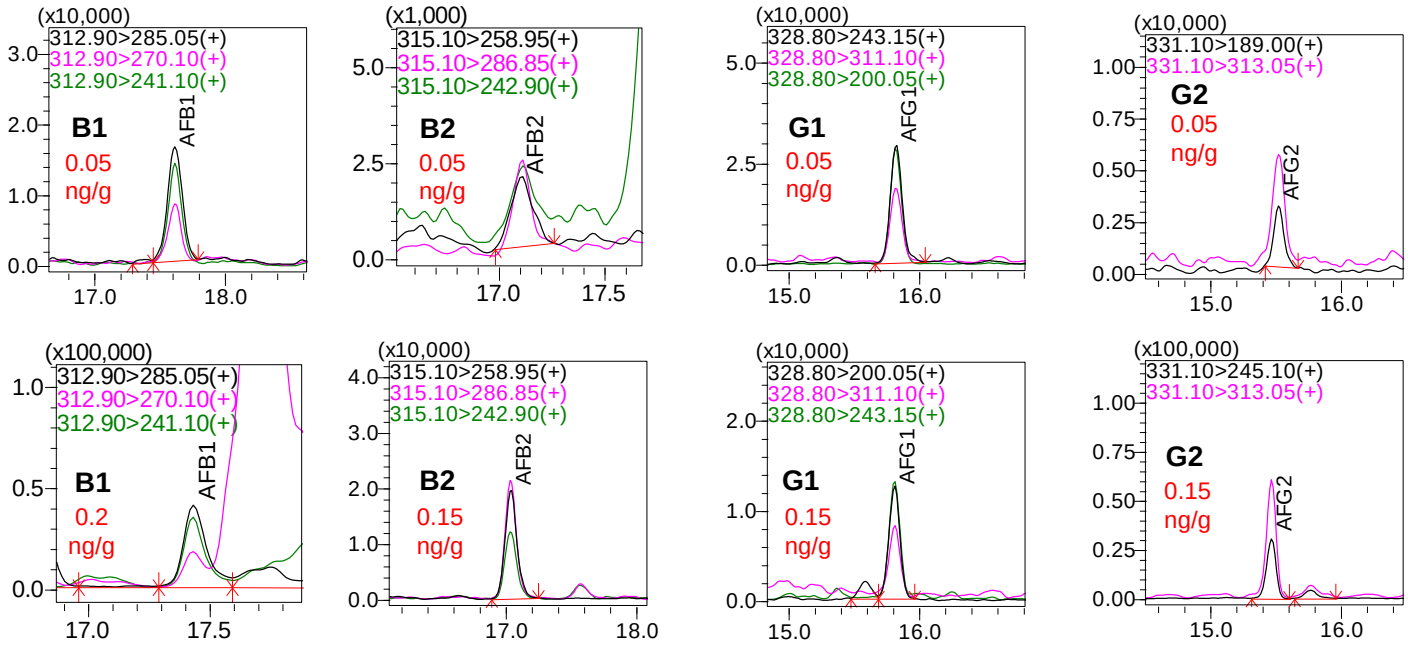

Figure 4. Representative individual MRM chromatograms of aflatoxin B1, B2, G1 and G2 spiked at respective LODs in maize (top) and peanut (bottom) in flour blank sample.

❑ Conclusion

An on-line SFE-SFC/MS/MS method for quantitation of aflatoxins in cereals and peanut flour was established and validated on Nexera UC. This method with using ^{13}C internal standards offers a fully-automated approach for screening of aflatoxin B1, B2, G1 and G2 in food samples without sample pre-treatment. Evaluated with certified reference materials, the method exhibits good extraction recovery (85.7-134%) and precision (RSD<15%). The aflatoxins values measured were within the assigned range. Although varying with different sample types, the detection limits of both peanut and cereal sample types are way below the regulatory limit. Hence, it is fit for the purpose of food safety screening analysis. By using $SFCO_2$, the Nexera UC offers a green approach in aflatoxins analysis.

❑ References

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