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Development of Automated Screening and Quantitation Approach on Novel On-Line SFE-SFC-MS/MS Platform – (II) For Five Aflatoxins in Powdered Food Samples

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Introduction

Supercritical fluid chromatography (SFC) is one of the trendy analytical techniques in recent years. By using carbon dioxide (CO_2) as eluent, the SFC has a number of advantages like environmental friendly, cost effective and suitable for a wide range of analytes' polarities. The Nexera UC system (Shimadzu) is a further developed platform combining SFE (extraction) and SFC with MS/MS into a complete system to handle from sample pre-treatment and separation to superior MS/MS detection and quantitation in a fully automated manner. The novel SFE-SFC-MS/MS system has been used successfully for analysis of 510 residual pesticides in agricultural products [1]. Here we describe the applications and advantages of the Nexera UC system in analysis of aflatoxins (B1, B2, G1, G2 and M1) in powdered food such as corn flour and wheat flour.

Experimental

Five aflatoxins (B1, B2, G1, G2 and M1) were obtained from Supelco and Romer Labs. Sixty mg of powdered sample (corn and wheat) were loaded into a 0.2 mL stainless steel vessel tightly before proceeding to on-line SFE-SFC-MS/MS. Spiked samples were prepared by soaking the powders in a desired amount of mixed standard solution and leaving it to dryness under N2 flow. A flow chart of the Nexera UC coupled with LCMS-8050 used in this study is shown schematically in Figure 1. A Shim-pack UC-X Sil column was used and a gradient elution program was adopted for analysis of the 5 aflatoxins. The detailed conditions are as shown in Table 1.

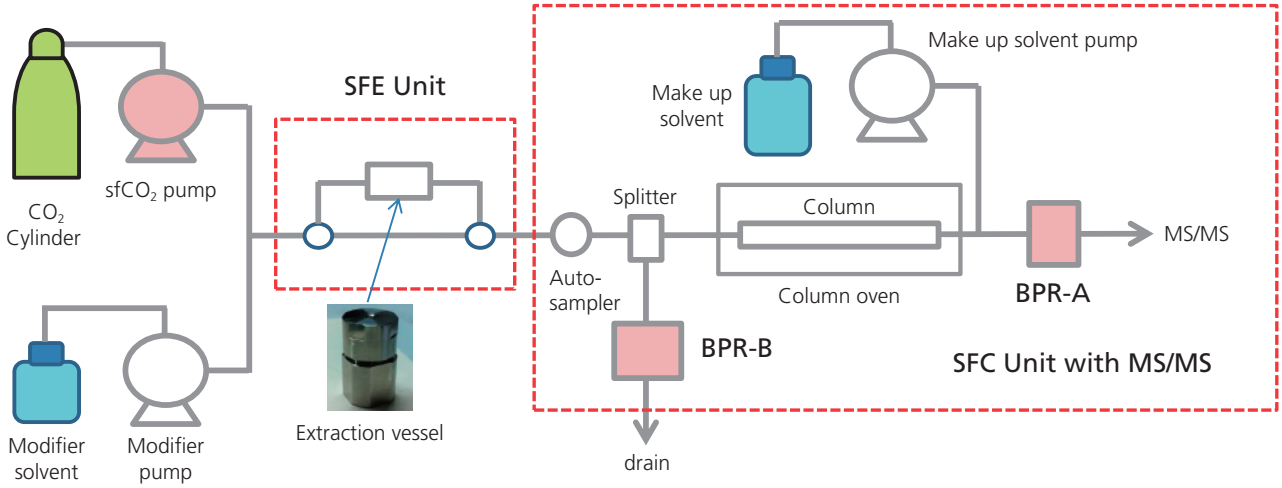

Figure 1: Schematic diagram of Nexera UC system for SFE-SFC-MS/MS analysis of un-pretreated samples

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Table 1: Analytical conditions of five aflatoxins on Nexera UC with LCMS-8050

Column	: Shim-pack UC-X Sil (250 mmL. x 2.1mm I.D., 3µm)
Flow Rate	: 2.0 mL/min
	0.2 mL/min (make up pump)
Mobile Phase	: A : Carbon dioxide
	B : Methanol with 5 mM ammonium formate
	C : Methanol with 0.1% formic acid
Oven Temp.	: Column oven: 40°C; SFE unit: RT
Injection vol.	: SFC: 5 µL; SFE-SFC: 200 µL
Elution Mode	: Gradient elution, LC program 5 minute
	0% B (0.00 mins to 0.50 mins)
	→ 40% B (3.00 mins to 3.50 mins)
	→ 0% B (3.60 mins to 5.00 mins)
Interface	: ESI
MS mode	: Positive, MRM
Block Temp.	: 400°C
DL Temp.	: 250°C
Interface Temp.	: 350°C
CID Gas	: Ar (270kPa)
Nebulizing Gas Flow	: N_2, 1.5 L/min
Drying Gas Flow	: N_2, 5.0 L/min
Heating Gas Flow	: 0 Air, 15.0 L/min

Results and Discussion

Establishment of SFC-MS/MS method for five aflatoxins

A SFC-MS/MS method for aflatoxin B1, B2, G1 G2 and M1 was established using mixed standard samples. Two MRM transitions were used for each aflatoxin compound, one as quantifier ion and the other for confirmation. Calibration curves with linearity (r^2>0.995) were obtained for all 5 aflatoxins (Table 2). Instead of using concentration for construction of the calibration curves, absolute amount (pg) was used (injection volume: 5uL). The LOQ of the SFC-MS/MS method ranges at 0.125 ~ 0.325 pg. The repeatability of the method was evaluated at two absolute amounts, 1.25 and 2.5 pg (B1, G1 and M1); 0.375 and 0.75 pg (B2 and G2). Six injections were made at every level of absolute amount for reliable results. The %RSD results of the mixed standards ranges from 4.0 ~ 9.5%, except for G2 at absolute amount of 0.375 pg where %RSD of 17.8% was obtained. The results of method performance are tabulated in Table 2.

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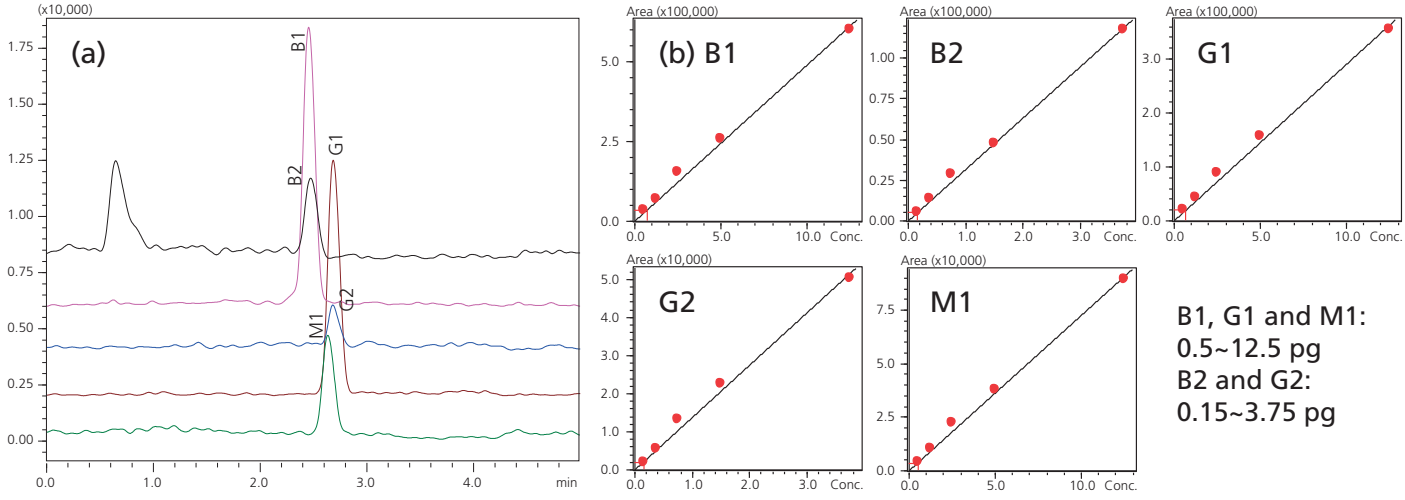

Figure 2: (a) MRM chromatograms of aflatoxins mixed standards B1, G1 and M1 at 1.25 pg; B2 and G2 at 0.375 pg.
(b) Calibration curves for aflatoxins B1, B2, G1, G2 and M1 in neat solution.

Table 2: Calibration curves and performance values of the MRM method for quantitation of five aflatoxins on SFC-MS/MS. Absolute amounts (in pg) of analytes are used for convenience (inj. volume: 5 µL)

Aflatoxin	Ret. Time	MRM transition	Cali. Range (pg)	r^2	Ave. Accuracy	LOQ (pg)	RSD (%), n=6			
							0.38 pg	0.75 pg	1.25 pg	2.5 pg
B1	2.452	313.1>241.1	0.5 - 12.5	0.998	100.2%	0.175	N.A.	N.A.	8.9	4.0
B2	2.473	315.1>287.0	0.15 - 3.75	0.998	102.6%	0.125	6.7	6.8	N.A.	N.A.
G1	2.698	329.1>243.1	0.5 - 12.5	0.998	100.5%	0.225	N.A.	N.A.	9.4	4.1
G2	2.693	331.1>245.1	0.15 - 3.75	0.995	99.8%	0.15	17.8	9.5	N.A.	N.A.
M1	2.637	329.2>229.1	0.5 - 12.5	0.997	101.9%	0.325	N.A.	N.A.	6.1	6.3

SFE-SFC-MS/MS method and system recovery

Based on the SFC-MS/MS method established above, on-line SFE-SFC-MS/MS was developed and evaluated. The mixed aflatoxin standard samples can be introduced into the system only by pre-loading them onto filter papers: 50 µL of mixed standard solution was dropped onto an half filter paper (recommended by Shimadzu) and left it to dryness under N2 flow before loading into the SFE vessel (0.2 mL). The results are shown in Figure 3 and Table 3. It can be seen that, with on-line SFE, the elution peaks of the aflatoxins become broader and RTs delay slightly in comparison with the SFC-MS/MS chromatograms. This peak broadening and delay (~0.5 mins) are due to the larger delay volume contributed by the SFE vessel, needles and the tubing from SFE to column, which caused differences in peak shape and intensity. For direct quantitation of PFCs using on-line SFE-SFC-MS/MS, calibration curves must be established on SFE-SFC-MS/MS too (Figure 3(b) & Table 3).

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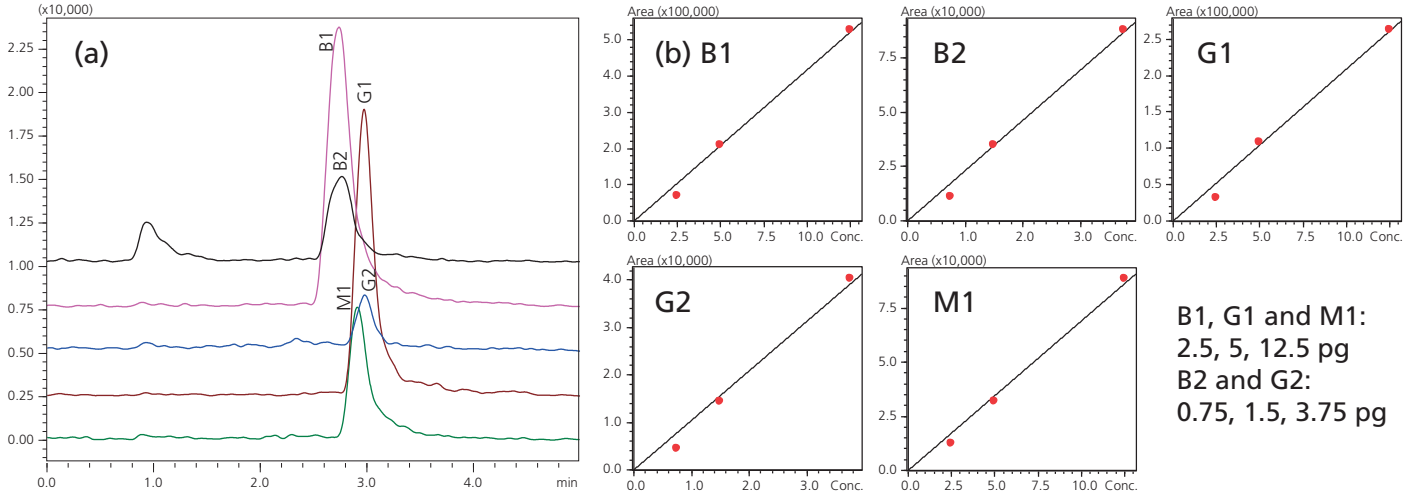

Figure 3: (a) MRM chromatograms of 5 aflatoxins on filter paper (B1, G1 and M1 at 5 pg; B2 and G2 at 1.5 pg).
(b) Calibration curves of aflatoxin B1, B2, G1, G2 and M1 on filter paper in SFE

Table 3: Calibration curves and performance values of MRM method for quantitation of five aflatoxins on SFE-SFC-MS/MS. Absolute amounts (pg) of analytes are used for convenience.

Aflatoxin	Ret. Time	MRM transition	Cali. Range (pg)	r^2	Ave. Accuracy	LOQ (pg)	RSD (%), n=6			
							0.75 pg	1.5 pg	2.5 pg	5 pg
B1	2.922	313.1>241.1	2.5 - 12.5	0.9963	98.6%	0.68	N.A.	N.A.	17.6	8.2
B2	2.949	315.1>287.0	0.75 - 3.75	0.9961	98.6%	0.59	11.0	5.7	N.A.	N.A.
G1	3.170	329.1>243.1	2.5 - 12.5	0.9920	97.9%	1.1	N.A.	N.A.	11.9	6.9
G2	3.169	331.1>245.1	0.75 - 3.75	0.9993	99.4%	2.5	17.5	11.8	N.A.	N.A.
M1	3.168	329.2>229.1	2.5 - 12.5	0.9989	99.2%	1.3	N.A.	N.A.	12.3	7.9

If we compare the peak areas obtained on SFE-SFC-MS/MS and SFC-MS/MS, system recovery of the SFE-SFC-MS/MS could be estimated. Although this system recovery may not be highly accurate, it can be used as a reference to understand the performance of the on-line SFE-SFC-MS/MS for quantitation. The system recovery measured with specific loading amounts are shown in Table 4. It is worth to note that all of the analysis runs shown above are under the condition without splitting of the flow ($sfCO_2$ and MeOH) from SFE to SFC-MS/MS.

Table 4: System recovery results of on-line SFE for aflatoxins

Aflatoxin	Ret. Time	MRM transition	Loaded (pg)	Measured (pg), n=3	System Recovery (%)
B1	2.452	313.1>241.1	5.0	4.30	86.0
B2	2.473	315.1>287.0	1.5	1.20	80.0
G1	2.698	329.1>243.1	5.0	3.80	76.0
G2	2.693	331.1>245.1	1.5	1.12	74.7
M1	2.637	329.2>229.1	5.0	4.45	89.0

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On-line SFE-SFC-MS/MS for analysis of aflatoxins in powdered food samples

There are two extraction modes in the on-line SFE stage, static extraction and dynamic extraction with $sfCO_2$ or a mixture of $sfCO_2$ and MeOH (modifier) [2]. In this work, only dynamic extraction of 2 mins was applied. A powdered corn flour sample (free of aflatoxins) was used to test the on-line SFE-SFC-MS/MS approach. The results of a representative analysis are shown in Figure 4 and Table 5. The spiked sample was prepared by dropping 50 µL of an aflatoxin stock solution onto 60 mg of the corn flour and left to dryness under N2 flow. Then, the sample was loaded into the 0.2 mL SFE vessel. It is noted that the measured amounts of aflatoxins in Table 5 are from the sum of two consecutive runs of the same sample. This is because the first run in a dynamic extraction mode could extract only less than 20 % of the spiked aflatoxins under the conditions. However, higher SFE extraction recovery is expected to be obtained if a static extraction step is used before the dynamic extraction.

The results of wheat flour (free of aflatoxins) samples spiked with aflatoxins are shown in Figures 5. It can be seen that more background peaks appeared in the chromatograms, which indicates that the matrix of wheat flour extracted by the on-line SFE is more complicated than the corn flour. This high backgrounds (relative) caused more difficulties to detect very low contents of aflatoxins in the sample. The results shown in Figure 5 suggest that the amounts of aflatoxins at 60~72 pg could be detected, which correspond to aflatoxin contents of 1.0~1.2 µg/kg in the spiked sample. Further studies on optimizing the SFE conditions to improve the on-line SFE recovery and reduce interference are on-going.

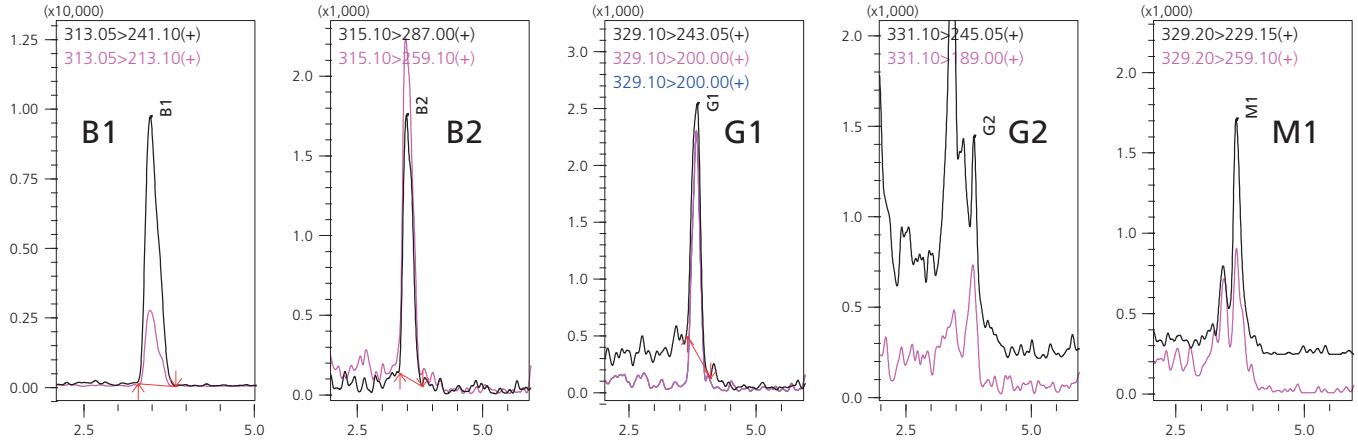

Figure 4: MRM peaks of aflatoxin detected in spiked corn flour sample detected in the 2nd run of the sample on SFE-SFC-MS/MS system. The spiked amounts of aflatoxins are shown in Table 5.

Table 5: Analysis results of aflatoxins spiked in powdered corn sample by on-line SFE-SFC-MS/MS approached

Aflatoxin	Spiked		Measured		Recovery (%)
	Abs (pg)	Cont (ug/kg)	Abs (pg)	Cont (ug/kg)	
B1	10.0	0.167	6.00	0.100	60.0
B2	3.0	0.050	1.84	0.031	61.3
G1	10.0	0.167	2.61	0.044	26.1
G2	3.0	0.050	0.71	0.012	23.7
M1	10.0	0.167	3.42	0.057	34.2

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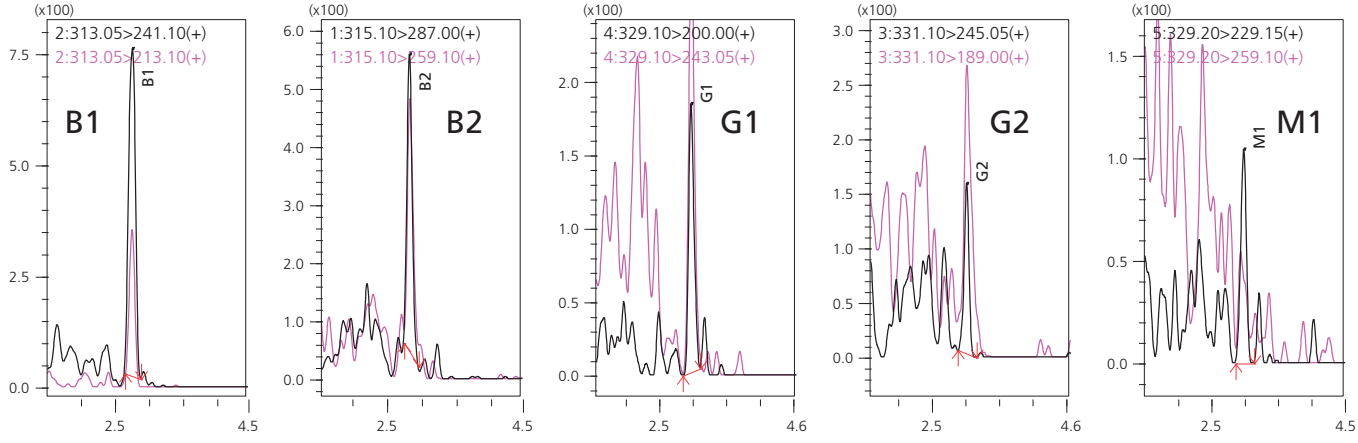

Figure 5: MRM peaks of aflatoxins spiked in wheat flour sample of 60 mg (B1, 60 pg, B2, 72 pg, G1, 60 pg, G2, 72 pg and M1, 60 pg) by on-line SFE-SFC-MS/MS.

Conclusions

A new analytical approach was developed on Shimadzu novel SFE-SFC-MS/MS platform for direct analysis of Aflatoxin B1, B2, G1, G2 and M1 in un-pretreated flour samples. The preliminary results indicate that this new approach is potentially applicable for screening and quantitation of aflatoxins in powdered food samples without any sample pre-treatment.

References

1. Shimadzu Application News No LAAN-A-LC-E273, Using the Nexera UC Online SFE-SFC-MS System to Analyze Residual Pesticides in Agricultural Products (2015).
2. J. Xing, , J. X. Lee, P. Zeng and Z. Zhan, Development of Automated Screening and Quantitation Approach on Novel On-line SFE-SFC-MS/MS Platform – (I) For 23 Restricted PFCs in Textiles; ASMS 2016, Poster Session MP 283.

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