APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

Authors:

PerkinElmer Applications Team

PerkinElmer, Inc.
Shelton, CT

Determination of Mycotoxins in Cereals by LC-MS/MS with Online SPE

Introduction

Mycotoxins are toxic secondary metabolites produced by fungi, and are capable of causing disease and death in both humans and animals. As such, many countries and regions have regulations and applicable permissible limits in place for mycotoxin detection and identification (Table 1).

Sample preparation is a critical step in the successful analysis of mycotoxins in food matrices. The "dilute and shoot" approach is a quick and easy way to introduce the sample into the LC/MS/MS analysis. However, due to the complexity of food matrices, this approach will often result in irreproducible matrix effects. Other sample prep techniques, such as offline solid phase extraction (SPE) and QUECHERS, require multiple time-consuming steps, and are prone to procedural and operator error.

To overcome these problems and improve sensitivity, this work utilizes the addition of online solid phase extraction (SPE), coupled to an LC/MS/MS system for sample enrichment. This approach allows for significant and efficient analyte concentration, obviating the need for elaborate and time-consuming sample preparation procedures.

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Table 1. Global maximum regulatory limits (µg/kg or ppb) for mycotoxins in processed cereal products intended for human consumption.

Mycotoxin	EU	USA	China	Singapore	Brazil
Sum of Aflatoxin B1, B2, G1 and G2	4	20	N/A	5	5
Aflatoxin B1 Only	2	N/A	5* / 20**	5	N/A
Sum of T-2 and HT-2 Toxins	75***	N/A	N/A	N/A	N/A
Sum of Fumonisin B1 and B2	800	N/A	N/A	N/A	400
Ochratoxin A	3	N/A	5*	3	10
Ergocristine	N/A	N/A	N/A	N/A	N/A
Zearalenone	75	N/A	N/A	N/A	N/A

* Grain products
** Corn/peanut products
*** Indicative level (regulatory level under discussion)
N/A = none available at the time

Experimental

Hardware/Software

For online analyte pre-concentration, enrichment and chromatographic separation, a PerkinElmer QSight® SP50 Online SPE System was used in combination with QSight® 210 MS/MS detector. All instrument control, analysis and data processing were performed using the Simplicity 3Q™ software platform.

Online SPE is accomplished through two additional six-port valves in the autosampler, and a High Pressure Dispenser (HPD). As shown in Figure 1, valve A is dedicated to SPE, while valve B allows for the flexible switching from direct injection to online SPE mode. The system was configured with a 10 µL stainless steel needle, a 1 mL sample loop, 1 mL syringe and 2 mL buffer tubing. Conditioning and equilibration solvents are delivered via the HPD, both solvents being directed to waste upon passing through the SPE cartridge. The sample is then aspirated into the sample loop using the autosampler syringe, and subsequently transferred via a load solvent from the loop to the SPE cartridge. This was followed by a wash step allowing for matrix components to be eluted off the cartridge. It should be noted that initial testing indicated that there were no significant analyte recovery differences with and without this rinse; however, to decrease the amount of matrix loaded, this step was included here. Analytes are then eluted off the SPE cartridge and onto the analytical column using the LC gradient. There is no separate SPE elution step needed, as the focused analytes on the SPE cartridge are eluted right onto the analytical column- as part of the chromatographic run.

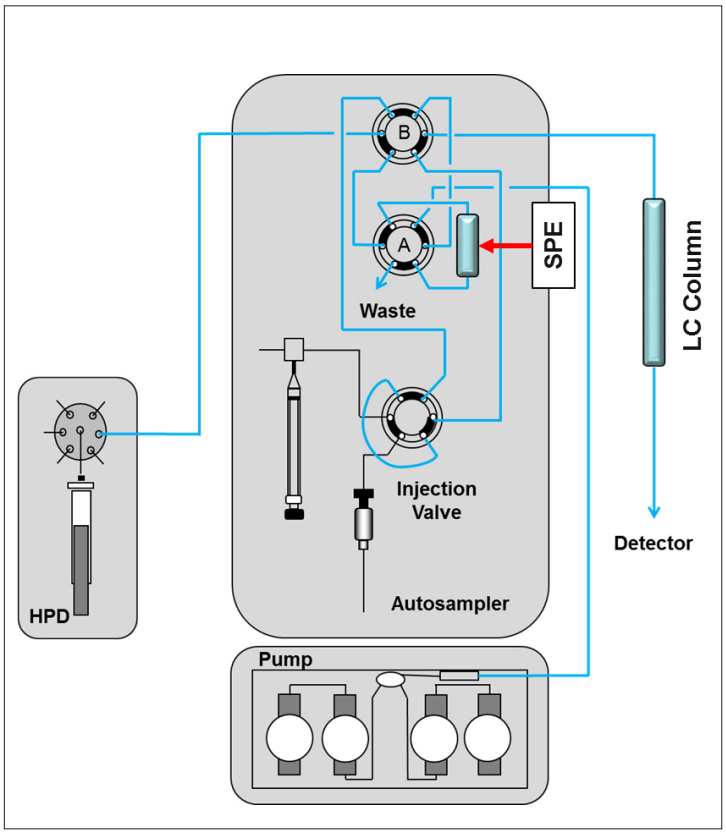

Figure 1. Schematic of QSight® SP50 Online SPE System.

For this method, sample enrichment was accomplished by loading a total of 1 mL of sample onto the SPE cartridge. The SPE parameters for this method are shown in Table 2.

A 24-vial tray was used, accommodating 10-mL sample vials (Part# N9300922; 100-vial/caps with integrated septa kit).

Method Parameters

The SPE, LC and MS/MS method parameters are shown in Tables 2-5.

Table 2. SPE Parameters.

SPE Cartridge	PerkinElmer Brownlee Spheri-5 C18, 5 µm, 2.1 x 30 mm (Part# 07110014)
SPE Solvents	SPE Solvent 1: Methanol with 0.1% formic acid SPE Solvent 2: 90:10 water/methanol with 0.1% formic acid SPE Solvent 3: 80:20 water/methanol with 0.1% formic acid
SPE Program	(Flow Rate = 1.5 mL/min for all steps)

Step	Step Type	Solvent 1 (mL)	Solvent 2 (mL)	Solvent 3 (mL)	Sample (mL)
1	Wash/Conditioning	2.0	-	-	-
2	Equilibration	-	2.0	-	-
3	Sample Loading into 1-mL Loop	-	-	-	1.5*
4	Sample Loading onto SPE cartridge	-	1.5	-	-
5	Wash	-	-	1.0	-

* The total sample load volume onto SPE cartridge is 1.0 mL (as fixed-loop injection mode is used, 0.5 mL of the 1.5-mL sample load goes to waste).

SPE Method Time	5.5 min. **Elution Time:** 4.0 min.

Table 3. LC Parameters.

Column	PerkinElmer Brownlee Analytical DB C18, 100 mm x 4.6 mm x 3 um (Part# N9303863)
SPE Solvents	Solvent A: Water with 0.1% formic acid and 5 mM ammonium formate Solvent B: 90/10 methanol/water with 0.1% formic acid and 5 mM ammonium formate Solvent Program:

Step	Time (min.)	Flow Rate (mL/min.)	%A	%B
1	0.0	1.00	45	55
2	3.5	1.00	45	55
3	3.75	1.00	10	90
4	7.0	1.00	10	90
5	7.1	1.00	45	55
6	11.0	1.00	45	55

Analysis Time	11.0 min
Oven Temp.	35 °C

Table 4. MS/MS Parameters.

Compound	ESI Mode	Ret. Time (min)	Time-Managed MRM™ Group	Precursor Ion	Frag. Ion 1 (Quantifier)	EV1	CCL2	CE1	Frag. Ion 2 (Qualifier)	EV1	CCL2	CE1
Aflatoxin G2	+	2.0	1.6 – 2.4 min	331.2	245.1	25	-125	-40	285.3	25	-120	-35
Aflatoxin G1	+	2.3	1.9 – 2.7 min	329.3	243.2	25	-110	-35	283.2	25	-75	-35
Aflatoxin B2	+	2.7	2.3 – 3.1 min	315.3	259.0	25	-95	-40	287.1	25	-90	-35
Aflatoxin B1	+	3.2	2.8 – 3.6 min	313.1	285.1	25	-100	-30	241.1	25	-125	-50
Fumonisin B1	+	5.0	4.6 – 5.4 min	722.4	352.3	25	-150	-45	334.5	25	-150	-50
HT-2 Toxin	+	5.0	4.6 – 5.4 min	447.1	345.1	25	-75	-25	285.1	25	-95	-25
Ergocristine	+	5.1	4.6 – 5.4 min	610.4	223.1	25	-120	-45	268.2	25	-120	-35
T-2 Toxin	+	5.2	4.8 – 5.6 min	489.2	245.2	25	-95	-35	387.1	25	-105	-25
Ochratoxin A	+	5.3	4.8 – 5.6 min	404.3	239.1	25	-90	-30	358.1	25	-75	-20
Fumonisin B2	+	5.4	5.0 – 5.8 min	706.3	336.4	25	-150	-45	318.3	25	-150	-50
Zearalenone	+	5.4	5.0 – 5.8 min	319.3	283.1	25	-55	-20	231.0	25	-60	-20

Table 5. MS/MS Source Parameters

Parameter	Value
Ionization Mode	ESI; Positive
Drying Gas	120
HSID Temperature (°C)	320
Nebulizer Gas	400
Electrospray Voltage (V)	4500
Source Temperature	400
Detection Mode	Time-Managed MRM™

Solvents, Standards and Samples

All solvents were LC-MS grade. The Aflatoxins mixture (B1, B2, G1, G2), HT-2 Toxin, T-2 Toxin, Ochratoxin A and Zearalenone standards were obtained from Sigma-Aldrich® (Zwijndrecht, The Netherlands). Ergocristine, Fumonisin B1 and Fumonisin B2 were obtained from Fermentek Ltd (Jerusalem, Israel) as powders. Stock solutions of Fumonisin B1 and B2 were prepared using LC-MS grade acetonitrile and a stock solution of Ergocristine was prepared in LC-MS grade methanol.

A working standard solution was then prepared from the stock standards, using 80:20 acetonitrile/water as the diluent. All calibrants were prepared via serial dilution from the working standard using the same diluent. Extracts were prepared by adding 20 mL of the 80:20 acetonitrile/water diluent to 5 g of homogenized cornflakes or multigrain cereal samples. The suspension was then shaken for 30 minutes, centrifuged for 10 minutes at 3500 rpm and decanted. The clear, yellowish supernatant was then spiked with corresponding calibrants. 1 mL of calibrants were added to 9 mL of extracts, resulting in a 10-fold dilution of the calibrants in extract. Prior to injection, 1 mL of the spiked extracts were further diluted with 9 mL of water for an additional 10-fold dilution. The final concentrations of mycotoxins in the injected samples were 40 times lower than corresponding levels in cornflakes/multigrain cereals (Table 6).

Results and Discussion

Figure 2 shows the chromatographic separation of the standard mixture of eleven analyzed mycotoxins. Working with analytes of very different polarities and physicochemical properties renders the separation challenging. This is seen in the chromatogram by the presence of two groups of analytes, more polar and early eluting Aflatoxins, and other less polar mycotoxins.

Table 6. Concentrations of analyzed mycotoxins in injected samples and corresponding range in cornflakes/multigrain cereals. 5 g of the samples were extracted with 20 mL of extraction solvent. Due to additional dilution of spiked extracts with water prior to injection (10 times) the final concentration in injected samples is therefore 40 times lower than would be in cornflakes/multigrain cereals.

Analyte	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7	Level 8	Level 9	Level 10	Range in Samples
Aflatoxin B1	0.0005	0.001	0.002	0.005	0.01	0.02	0.05	0.1	0.2	0.5	0.02 – 20
Aflatoxin B2	0.0005	0.001	0.002	0.005	0.01	0.02	0.05	0.1	0.2	0.5	0.02 – 20
Aflatoxin G1	0.0005	0.001	0.002	0.005	0.01	0.02	0.05	0.1	0.2	0.5	0.02 – 20
Aflatoxin G2	0.0005	0.001	0.002	0.005	0.01	0.02	0.05	0.1	0.2	0.5	0.02 – 20
Ergocristine	0.005	0.01	0.02	0.05	0.1	0.2	0.5	1	2	5	0.2 – 200
Fumonisin B1	0.2	0.4	0.8	2	4	8	20	40	80	200	8 – 8000
Fumonisin B2	0.2	0.4	0.8	2	4	8	20	40	80	200	8 – 8000
HT-2 Toxin	0.002	0.004	0.008	0.02	0.04	0.08	0.2	0.4	0.8	2	0.08 – 80
Ochratoxin A	0.005	0.01	0.02	0.05	0.1	0.2	0.5	1	2	5	0.2 – 200
T-2 Toxin	0.002	0.004	0.008	0.02	0.04	0.08	0.2	0.4	0.8	2	0.08 – 80
Zearalenone	0.02	0.04	0.08	0.2	0.4	0.8	2	4	8	20	0.8 – 800

Level 1 – Level 10: concentrations in injected samples. All concentrations expressed in ppb or ng/mL.

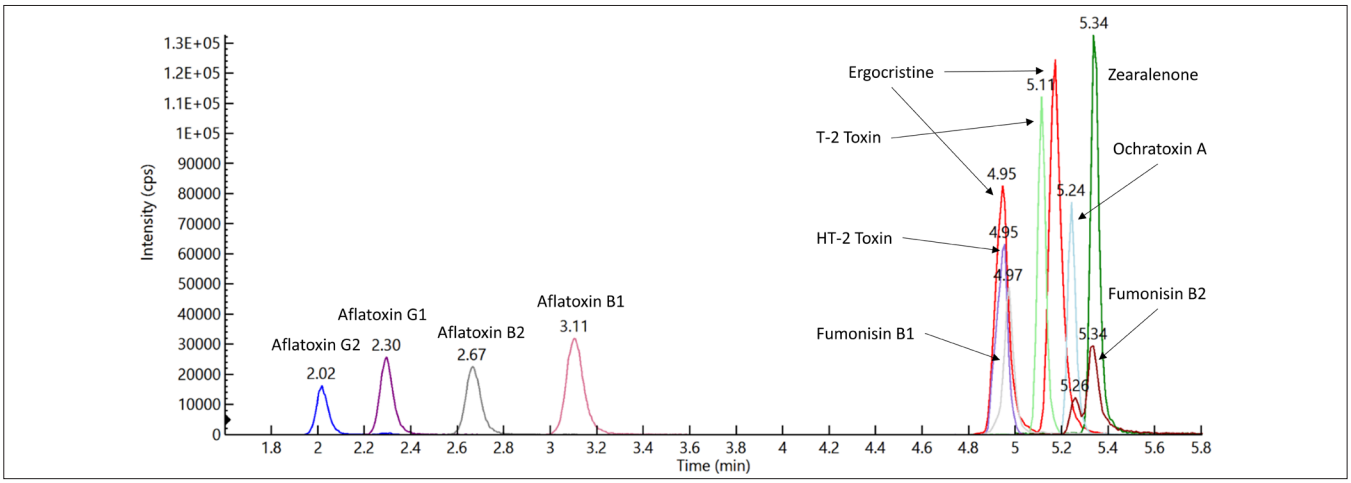

Figure 2. Overlay MRM chromatograms, showing the separation of the sample containing all 11 analyzed mycotoxins.

Two peaks are observed for Ergocristine as it is present in solution as a mixture of two epimers (the two isomers differ in configuration at only one stereogenic center). These isomers are chromatographically separated but in this analysis account for one compound. Since the ratio between the two epimers could vary, integration of both peaks in unison is used for the quantitation. Per Figure 3, chromatographic repeatability was found to be exceptional, here shown via the chromatographic overlay of ten replicate mycotoxins mixture injections for each analyte separately.

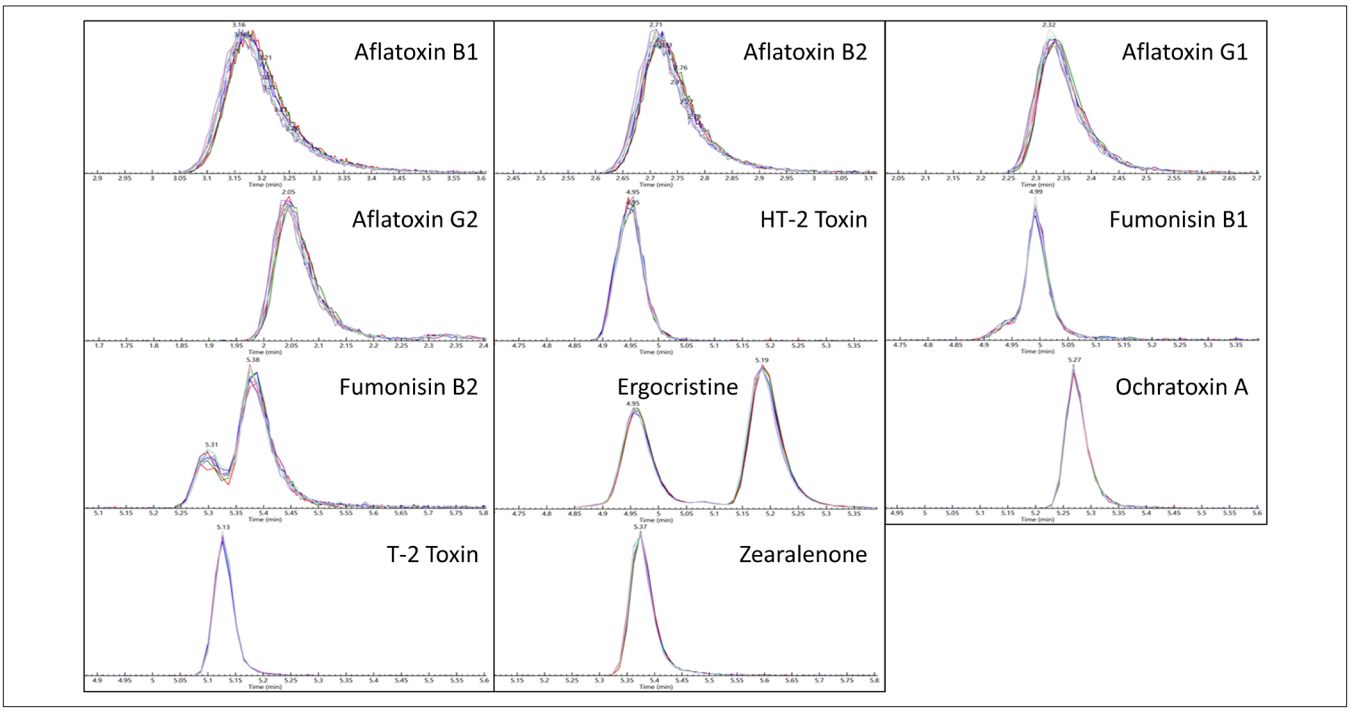

Figure 3. Overlay of ten replicate injection of the 11 mycotoxins spiked into extraction solvent (acetonitrile/water, 80/20).

The linearity plots for the four selected mycotoxins in extracts are shown in Figure 4, with R^2 values all above 0.995. The inserts show very good linearity even at lower end of the calibration range. This demonstrates that the calibration curves have a linear range over several orders of magnitude that extend significantly below regulated limits (Tables 1 and 6) but also above them.

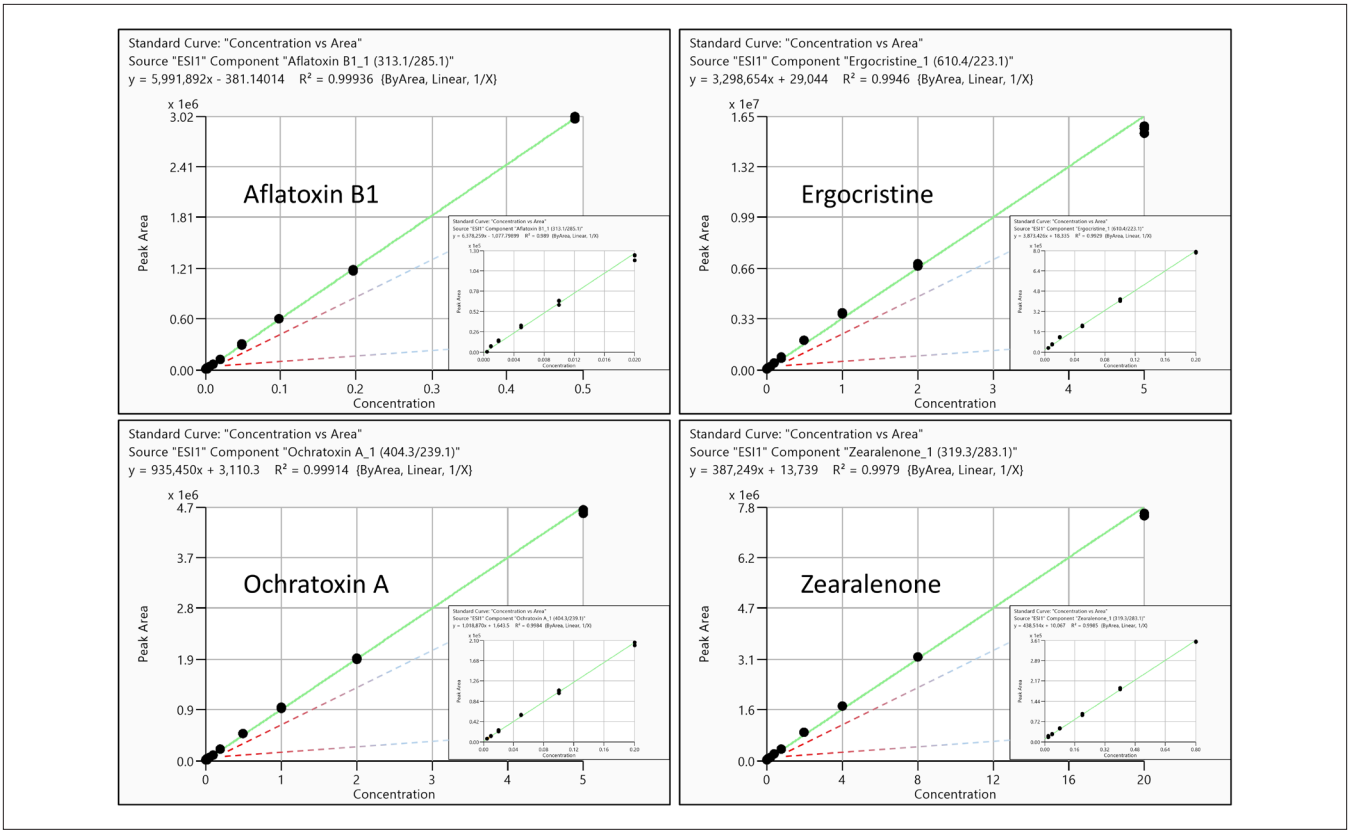

Figure 4. Linearity plots for the four selected mycotoxins. Inserts show linearity plots for the lower end of the concentration range.

As listed in Table 7, limits of quantitation (LOQs) were established for each mycotoxin, based upon their averaged Level 1 calibration standard response. Limits of quantitation are well below maximum regulatory limits. The representative MRM chromatograms of Level 1 calibration standard are shown in Figure 5.

Table 7. Calculated LOQs for the eleven analytes.

Analyte	Calculated LOQ (ppb)	Analyte	Calculated LOQ (ppb)
Aflatoxin B1	0.010	Fumonisin B2	9.863
Aflatoxin B2	0.013	HT-2 Toxin	3.198
Aflatoxin G1	0.011	Ochratoxin A	0.157
Aflatoxin G2	0.034	T-2 Toxin	0.146
Ergocristine	0.014	Zearalenone	0.890
Fumonisin B1	2.547		

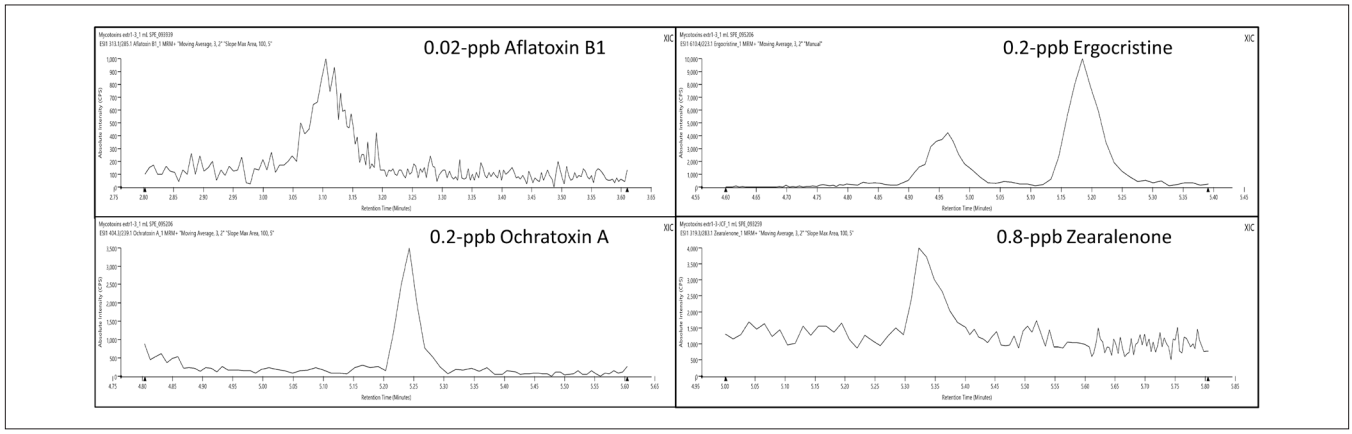

Figure 5. MRM chromatograms of the Level 1 calibration standard for selected mycotoxins

Figure 6 shows the results of the 50-µL direct injection (top) compared to 1000-µL SPE loading (bottom) of Level 1 calibration standard for selected analytes. Using a 50-µL direct injection, only Ergocristine can be reliably detected in the spiked cereal extract. By comparison, the 1000-µL SPE load allows for the detection and quantitation of all four selected mycotoxins at Level 1 calibration standard in cereal extract, with a 10-fold increase in peak area for Ergocristine.

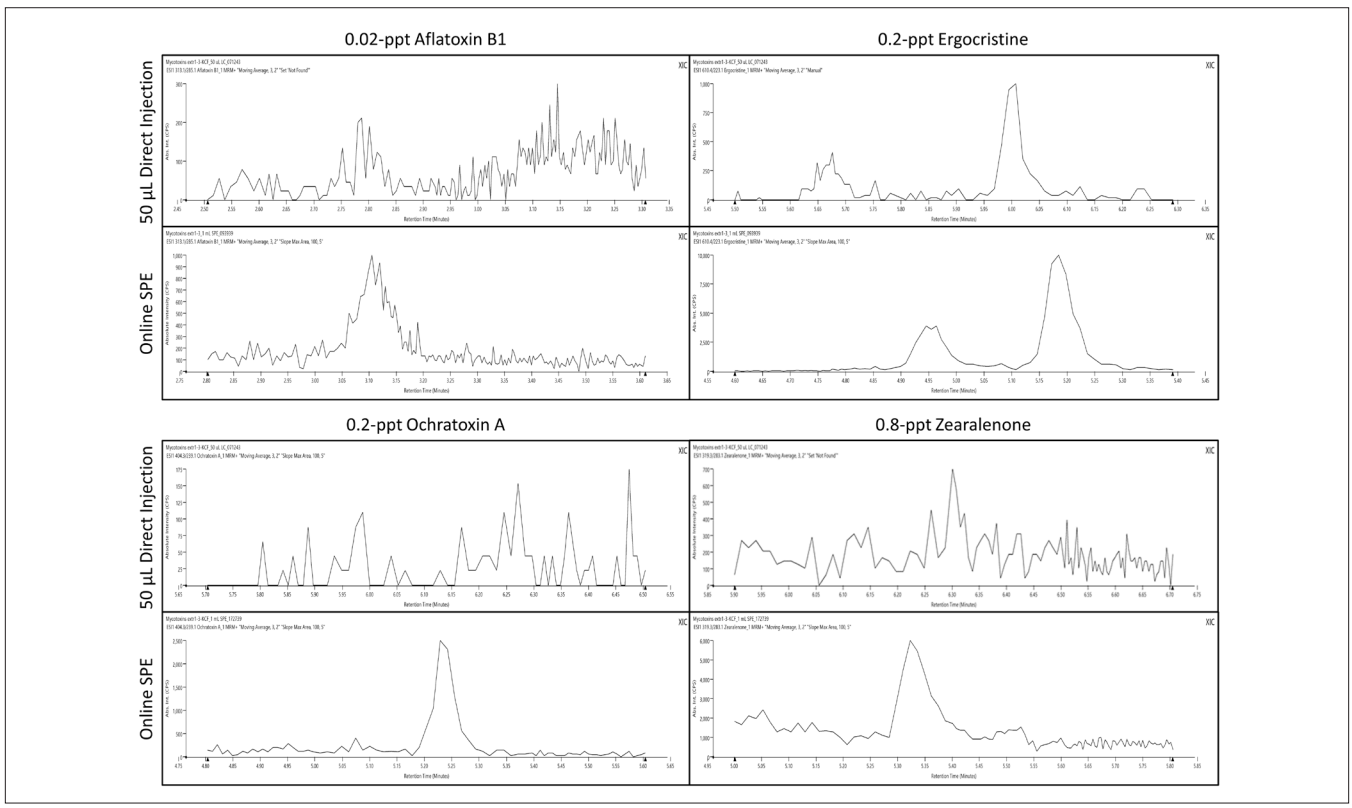

Figure 6. Comparative results of Level 1 calibration standard with 50-µL direct injection (top) and 1000uL SPE loading (bottom), both in cornflakes extract, for selected mycotoxins.

Taking Aflatoxin B1 as an example, sample loading repeatability, recovery performance and reliable analyte confirmation are demonstrated in Table 7, per *Sample Accuracy % (Area), CV%* (Area) and *Ion Ratio* columns, across all calibrant levels. As highlighted in green, all values were well within acceptable tolerances. Though not shown, the results for other mycotoxins were equally impressive. As seen at the top of the table, the results also confirmed the absence of any detectable Aflatoxin B1 in the analyzed cornflakes sample. This was not the case for all of the mycotoxins in the six different analyzed samples, as shown in Table 8, where all the detected mycotoxins are listed. Nevertheless, all the detected mycotoxins were significantly below the EU regulated levels, which are the most demanding.

Table 7. Sampling performance and analyte confirmation results for Aflatoxin B1.

	Sample	Peak Area	Known Concentration	Concentration by Area	Sample Accuracy % (Area)	CV% (Area)	Ion Ratio (241.1/285.1) Area	Expected Ion Ratio	Signal to Noise Ratio	Retention Time Deviation %
1	Blank_1 mL SPE	Not Found		N/A	N/A	N/A	N/A	0.60, 0.90	N/A	N/A
2	Blank_1 mL SPE	Not Found		N/A	N/A	N/A	N/A	0.60, 0.90	N/A	N/A
3	Blank_1 mL SPE	Not Found		N/A	N/A	N/A	N/A	0.60, 0.90	N/A	N/A
4	Blank extract_1 mL SPE	Not Found		N/A	N/A	N/A	N/A	0.60, 0.90	N/A	N/A
5	Mycotoxins extr1-3_1 mL SPE	4575	0.0005	0.000	97.102	0.802	0.71	0.60, 0.90	19.253	0.428
6	Mycotoxins extr1-3_1 mL SPE	4507	0.0005	0.000	94.801	0.802	0.62	0.60, 0.90	26.308	0.151
7	Mycotoxins extr1-3_1 mL SPE	4564	0.0005	0.000	96.729	0.802	0.62	0.60, 0.90	19.329	0.151
8	Mycotoxins extr1-2_1 mL SPE	7162	0.001	0.001	92.305	1.474	0.68	0.60, 0.90	37.683	0.299
9	Mycotoxins extr1-2_1 mL SPE	7333	0.001	0.001	95.197	1.474	0.79	0.60, 0.90	41.304	0.074
10	Mycotoxins extr1-2_1 mL SPE	7360	0.001	0.001	95.654	1.474	0.79	0.60, 0.90	57.256	0.151
11	Mycotoxins extr1-1_1 mL SPE	13101	0.002	0.002	96.376	4.156	0.75	0.60, 0.90	65.133	0.151
12	Mycotoxins extr1-1_1 mL SPE	13733	0.002	0.002	101.720	4.156	0.69	0.60, 0.90	96.459	0.074
13	Mycotoxins extr1-1_1 mL SPE	12644	0.002	0.002	92.511	4.156	0.81	0.60, 0.90	91.248	0.376
14	Mycotoxins extr1_1 mL SPE	31520	0.005	0.005	100.855	2.395	0.79	0.60, 0.90	168.736	0.151
15	Mycotoxins extr1_1 mL SPE	33060	0.005	0.005	106.064	2.395	0.74	0.60, 0.90	198.707	0.151
16	Mycotoxins extr1_1 mL SPE	32435	0.005	0.005	103.950	2.395	0.76	0.60, 0.90	137.337	0.074
17	Mycotoxins extr2_1 mL SPE	65692	0.01	0.011	108.223	2.858	0.73	0.60, 0.90	428.432	0.151
18	Mycotoxins extr2_1 mL SPE	62233	0.01	0.010	102.372	2.858	0.78	0.60, 0.90	212.507	0.074
19	Mycotoxins extr2_1 mL SPE	62989	0.01	0.010	103.651	2.858	0.74	0.60, 0.90	271.627	0.299
20	Mycotoxins extr3_1 mL SPE	123328	0.02	0.021	102.851	0.695	0.79	0.60, 0.90	513.842	0.151
21	Mycotoxins extr3_1 mL SPE	123185	0.02	0.021	102.730	0.695	0.74	0.60, 0.90	423.155	0.151
22	Mycotoxins extr3_1 mL SPE	124742	0.02	0.021	104.047	0.695	0.76	0.60, 0.90	637.597	0.151
23	Mycotoxins extr4_1 mL SPE	304027	0.05	0.051	102.264	0.773	0.77	0.60, 0.90	583.377	0.074
24	Mycotoxins extr4_1 mL SPE	308239	0.05	0.052	103.689	0.773	0.75	0.60, 0.90	1847.276	0.151
25	Mycotoxins extr4_1 mL SPE	304281	0.05	0.051	102.350	0.773	0.77	0.60, 0.90	790.508	0.151
26	Mycotoxins extr5_1 mL SPE	583357	0.1	0.098	98.375	0.665	0.75	0.60, 0.90	1120.041	0.151
27	Mycotoxins extr5_1 mL SPE	585694	0.1	0.099	98.770	0.665	0.75	0.60, 0.90	1043.946	0.151
28	Mycotoxins extr5_1 mL SPE	590977	0.1	0.100	99.664	0.665	0.74	0.60, 0.90	963.483	0.151
29	Mycotoxins extr6_1 mL SPE	1170516	0.2	0.198	98.841	0.352	0.76	0.60, 0.90	791.497	0.151
30	Mycotoxins extr6_1 mL SPE	1177542	0.2	0.199	99.435	0.352	0.76	0.60, 0.90	1104.355	0.074
31	Mycotoxins extr6_1 mL SPE	1177801	0.2	0.199	99.457	0.352	0.76	0.60, 0.90	1018.907	0.074
32	Mycotoxins extr7_1 mL SPE	2935059	0.5	0.496	99.224	0.772	0.76	0.60, 0.90	1154.156	0.299
33	Mycotoxins extr7_1 mL SPE	2980721	0.5	0.504	100.768	0.772	0.76	0.60, 0.90	1139.773	0.428
34	Mycotoxins extr7_1 mL SPE	2958781	0.5	0.500	100.026	0.772	0.76	0.60, 0.90	998.061	0.524
35	Blank_1 mL SPE	Not Found		N/A	N/A	N/A	N/A	0.60, 0.90	N/A	N/A
36	Blank_1 mL SPE	Not Found		N/A	N/A	N/A	N/A	0.60, 0.90	N/A	N/A
37	Blank_1 mL SPE	Not Found		N/A	N/A	N/A	N/A	0.60, 0.90	N/A	N/A

Table 8. Mycotoxins detected in analyzed samples and the comparison to the EU regulated levels.

Analyte	Extract 1	Extract 2	Extract 3	Extract 4	Extract 5	Extract 6	EU Regulations	
							Individual	Sum
Aflatoxin B1							2	4
Aflatoxin B2							N/A	
Aflatoxin G1						0.01	N/A	
Aflatoxin G2						0.01	N/A	
Ergocristine					0.07	0.04	N/A	
Fumonisin B1							N/A	800
Fumonisin B2							N/A	
Ochratoxin A						0.03	3	
HT-2 Toxin			1.43		1.8	1.64	N/A	75
T-2 Toxin			1.58			1.41	N/A	
Zearalenone	0.97		3.03	0.07			75	

All concentrations expressed in ppb (ng/mL). N/A = none available at the time.

To evaluate matrix effects from cornflakes and multigrain cereals, calibration curves of these extracts spiked with corresponding concentrations of mycotoxins were compared to calibration curves prepared neat in solvent. Even though matrix effects for some analytes were significant (Table 9), a comparison of six different extracts from different cornflakes and multigrain cereals showed little variation in the amount of matrix effects from sample to sample. Matrix effects were calculated by comparison of the slope coefficients from analytes spiked in extracts and analytes spiked neat into solvent (Figure 7). As the matrix effects do not differ significantly between different sample extracts and between different concentrations of analytes (Table 9), and good linearity over several orders of magnitude was achieved, matrix-matched calibration curves are recommended as a viable and robust way to compensate for matrix effects in the analysis of mycotoxins in cornflakes and multigrain cereals.

Table 9. R^2 values for linearity plots and matrix effect for mycotoxins spiked to six different cornflakes and multigrain cereals extracts. Matrix effects are shown as the average over six extracts with a separate column showing standard deviation. Matrix effects are expressed as the signal in extract divided by the signal in neat solvent. Matrix effects below 100% indicate ion suppression, while matrix effects above 100% indicate ion enhancement.

Analyte	Extract 1	Extract 2	Extract 3	Extract 4	Extract 5	Extract 6	Matrix effect (%)	STDEV (%)
Aflatoxin B1	1.000	0.999	1.000	0.999	0.999	1.000	60	5
Aflatoxin B2	1.000	0.999	0.998	0.999	0.999	1.000	57	5
Aflatoxin G1	1.000	0.999	0.999	1.000	1.000	1.000	48	8
Aflatoxin G2	1.000	0.999	1.000	1.000	0.998	1.000	43	6
Ergocristine	0.996	0.997	0.987	0.992	0.994	0.998	51	11
Fumonisin B1	0.999	0.999	0.998	0.999	0.998	0.998	135	6
Fumonisin B2	0.996	0.997	0.995	0.998	0.997	0.996	128	7
HT-2 Toxin	0.985	0.993	0.994	0.992	0.960	0.979	3	2
Ochratoxin A	0.999	0.999	0.998	0.999	1.000	1.000	87	5
T-2 Toxin	0.993	0.985	0.999	0.982	0.988	0.987	13	5
Zearalenone	0.998	0.998	0.998	0.998	0.999	0.998	54	7

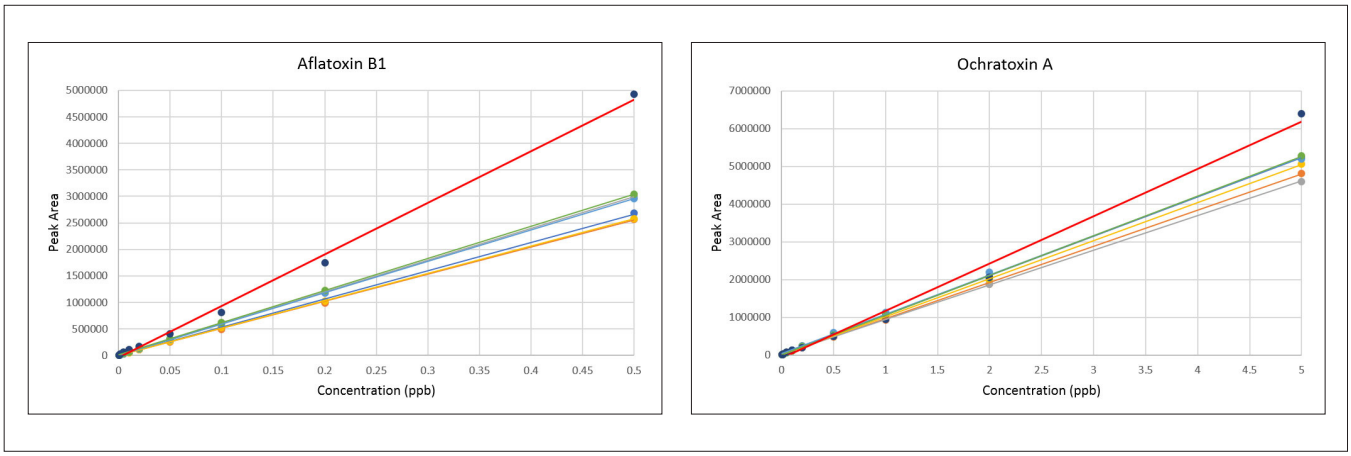

Figure 7. Ten-point calibration curves for Aflatoxin B1 and Ochratoxin A. Calibration curves in pure solvent (80/20 acetonitrile/water) are shown in red. Calibration curves for extracts from five different cornflakes and one multigrain cereal all show very similar results. Comparison of the slope coefficients gives the matrix effect as shown in Table 9.

Conclusions

- This work has demonstrated the effective and robust online SPE sample loading and chromatographic separation/quantitation of mycotoxins, using a PerkinElmer QSight SP50 Online SPE System with a QSight 210 MS/MS detector.
- Due to the unique high-capacity SPE cartridge and the high enrichment factor, large sample sizes are not required, saving time, reducing laboratory waste and improving throughput while simultaneously increasing the sensitivity of a standard LC-MS/MS system.
- As the Simplicity 3Q software automatically provides for work-ahead flow as part of the online SPE/chromatographic process, the total time necessary for sample preparation and analysis is reduced to just 12.5 minutes per sample.
- This procedure allows for relatively low solvent consumption (≤10 mL per sample) as part of the SPE preparation phase.
- The method provides exceptional online sample preparation/pre-concentration and chromatographic repeatability, and affords LOQs significantly below regulated levels.
- Matrix-matched, ten-point calibration curves were obtained for simultaneous analysis of eleven mycotoxins. Excellent linearity was achieved for all the mycotoxins in the concentration ranges over three orders of magnitude.
- The method/procedure defined herein can be expected to fulfill the essential task of monitoring for low-level mycotoxins in cornflakes and multigrain cereals.

References

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PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer®

For a complete listing of our global offices, visit www.perkinelmer.com/ContactUs

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