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Development and in-house Validation of a Rapid LC-MS/MS Method for the Semi-quantification of Eleven Mycotoxins in Maize Samples

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Abstract: The aim of this study is a current trend in chemical food safety control to increase monitoring of the co-occurrence of mycotoxins. Unsanitary conditions during harvesting, drying, packing and storage in production and processing of cereals can effect mycotoxin contamination. A method was developed for the simultaneous determination of 11 mycotoxins: deoxynivalenol, aflatoxins B1, B2, G1 and G2, ochratoxin A, fumonisins B1 and B2, zearalenone, and toxins T-2 and HT-2, allowing confirmation of their presence in maize samples as well as their identification and semi-quantification. The mycotoxins are extracted with a mixture of methanol/water, diluted with water and 0.1% formic acid and then analyzed by LC-HESI-MS/MS in a single 12 min run in positive mode. Multiple reaction monitoring mode (MRM) is applied by using two abundant fragments for each mycotoxin. Matrix effects are compensated using external matrix-matched calibration curves. Recoveries, calculated by spiking blank maize samples, ranging from 95.2% to 113.4%, were in accordance with the performance criteria required by the European Commission and intraday reproducibility ranged from 4.2% to 13.2%. Proficiency test materials and reference materials were analyzed to assess the accuracy of the method with satisfying results for the 11 mycotoxins. The method was used for monitoring of maize samples on sale in Switzerland.

Keywords: Cereals · LC-MS/MS · Maize · Mycotoxins

1. Introduction

One of the aspects of providing food quality is the public, *i.e.* consumer health. Over the past years food safety has focused on the finished goods as well as the production system. Close attention is focused on cereals and their products as they are considered to be staples in many countries. Mycotoxins are secondary metabolites produced by molds that can grow on agricultural commodities, such as cereals and other crops, during harvesting, drying, packing and storage in production and processing. A multiple contamination can be present since some molds can produce more than one mycotoxin and some mycotoxins can be produced by more than one fungal species.[1] Mycotoxins are chemical and thermal stable compounds, so they can survive during processing steps.[2] Due to the toxicity of these compounds, regular monitoring and surveillance is important. For this reason Swiss and European maximum legal limits have been set up[3,4] for nine mycotoxins (deoxynivalenol, aflatoxins B1, B2, G1 and G2, ochratoxin A, fumonisins B1 and B2 and zearalenone) while for toxins T-2 and HT-2 the European Recommendation (2013/165/EU) for collecting data on the presence of these toxins in cereals could lead to a regulation in the near future.

The current conventional methods for the determination of these mycotoxins in low concentrations rely on high performance liquid chromatography (HPLC) coupled with a UV or fluorescence detector[5–8] and more recently mass spectrometry detection (MS). Different strategies also exist for the sample preparation and clean up before mycotoxin analysis. The use of selective antibodies (immunoaffinity columns) is widely known for the isolation and purification from different matrices. The use of the MS detector offers high sensitivity, unambiguous analyte identification, accurate quantification and it has become an important tool for multiresidue analysis by providing a selective detection of analytes. Nowadays, methods like enzyme-like immunosorbent assays (ELISAs), fluorescence polarization immunoassays and biosensors also provide rapid screening of samples but are still qualitative tools. The recent trend is to use the HPLC–tandem mass spectrometry (MS/MS) method by injecting a diluted crude extract sample for the simultaneous quantification of mycotoxins without any purification step taking into account the different physicochemical properties of mycotoxins.[9,10]

The aim of this work is to develop and validate a simple method for the analysis of the 11 mycotoxins with different chemical structures (Fig. 1) in maize samples by using tandem mass spectrometric detection with electrospray ionization in order to facilitate a rapid estimation of the presence of mycotoxins in a large number of cereal samples, which can provide a cost-effective and rapid monitoring of food contamination for official controls.

2. Materials and Methods

2.1 *Chemicals and Reagents*

Methanol HPLC grade for extraction (Honeywell) was purchased from Burdick & Jackson, ammonium bicarbonate (BioUltra) was obtained from Fluka (Switzerland) and water was purified by an Elga Labwater ultra genetic (Labtec

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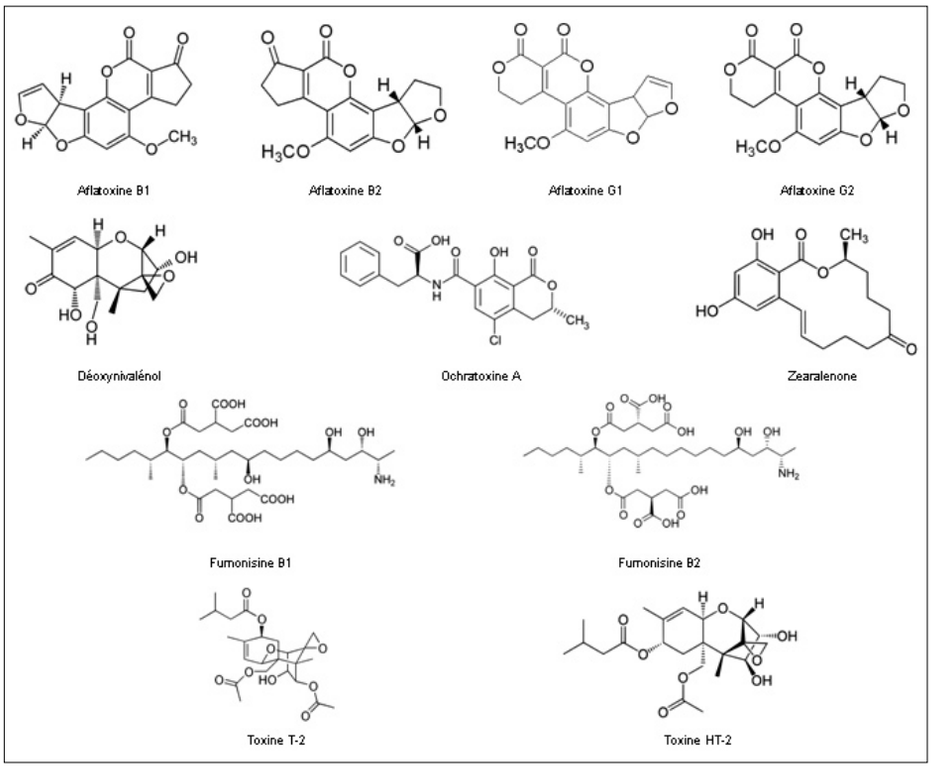

Fig. 1. Mycotoxins in the present study.

Services AG, Switzerland). Methanol, water with 0.1% formic acid and formic acid for LC-MS mobile phase were purchased from Fisher Scientific (Switzerland).

Stock solutions of aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2), deoxynivalenol (DON), ochratoxin A (OTA), zearalenone (ZEA), and toxins T-2 and HT-2 (all in acetonitrile) and fumonisins B1 (FB1) and B2 (FB2) (in acetonitrile:water 1:1) were purchased from Biopure (Tulln, Austria).

The following CRMs (certified reference materials) and FAPAS® testing materials were used for trueness of method: (i) Trilogy®CRM corn naturally contaminated with mycotoxins (Lot # MTC-9999E, Trilogy analytical laboratory, Washington) (DON: 2600.00±447.20 µg/kg, AFB1: 18.80±6.58 µg/kg, AFB2: 0.90±0.32 µg/kg, AFG1: 2.40±0.84 µg/kg, AFG2: non detected ±35%, FB1: 28300.00±7584.40 µg/kg, FB2: 7100.00±1902.80 µg/kg, ZEA: 352.00±112.64 µg/kg, OTA: 4.00±3.46 µg/kg, T-2: 263.70±121.83 µg/kg, HT-2: 523.30±173.74 µg/kg) and (ii) FAPAS® proficiency testing materials no. 04201 (DON: 2013.00 µg/kg, AFB1: 6.80 µg/kg, ZEA: 396.00 µg/kg, OTA: 8.34 µg/kg), 04214 (DON: 1329.00 µg/kg , AFB1: 13.20 µg/kg, ZEA: 290.00 µg/kg, OTA: 8.89 µg/kg) and 04223 (DON: 1247.00 µg/kg, AFB1: 5.42 µg/kg, FB1: 797.00 µg/kg, FB2: 360.00 µg/kg, ZEA: 286.00 µg/kg, OTA: 2.40, T-2: 160.00 µg/kg, HT-2: 105.00 µg/kg) obtained from the Food and Environmental Research Agency (FERA, York, UK).

2.2 *Extraction*

Blank or low contaminated maize samples of 1 kg were collected from local retail outlets in Switzerland in the area of the canton of Vaud. The samples were milled in fine flour and homogenized by using an Inversina Tumbler Mixer (Bioengineering, Switzerland). 10 g of a representative sample was weighted in a 100 mL Erlenmeyer flask with 2 mg of ammonium bicarbonate and shaken vigorously for 10 min with 40 ml methanol:water (80:20, v/v). These extracts were then sonicated for 10 min and then filtered through a Whatman SS 595 ½ pleated filter. The 2 mL of the extract was diluted with water 0.1% formic acid in 1:2 (v/v) ratio. Prior to final instrumental analysis, the sample solution was filtered through 0.45 µm filter.

2.3 *LC-MS/MS System and Conditions*

The system Accela 1250 LC-QqQ-MS/MS Vantage (Thermo Fisher Scientific, USA) was used for the mycotoxin analysis equipped with a heated electrospray ionization probe (HESI). The column of the ultra-high performance liquid chromatography was a Hypersil GOLD 100×2.1 mm, 3 µm (Thermo Fisher Scientific, USA) with a pre-column. The column temperature was kept at 30 °C and the flow rate was at 0.25 mL/min. The injection volume was 10 µL. The mobile phase consisted of eluent A containing water and eluent B consisting of methanol. Both eluents contained 0.1% formic acid. A gradient program was used for the chromatographic separation of mycotoxins. This program started with 95% A and 5% B and after 1 min a linear gradient was applied reaching 70% B after 9 min and then switched back (9.10 min) to 95% A which was maintained until the end of the run at 12 min. The parameters of the source were as follows: spray voltage at 3 kV, vaporizer temperature at 200 °C, sheath gas pressure at 30 arbitrary units, auxiliary gas pressure at 10 arbitrary units, and capillary temperature at 280 °C. Nitrogen was used as the collision gas. The other parameters were automatically tuned for each analyte separately. LC-MS/MS parameters used for the separation and identification of mycotoxins are presented in Table 1.

The compounds were detected in MS/MS analyzer using MRM mode. Two ion transitions are selected for each compound, a quantification ion and a confirmatory ion. The MS/MS detection included four time segments, in which different ionization conditions and ion transitions are set with a dwell time of 0.03 s for each compound.

Table 1. LC-MS/MS parameters of mycotoxins under optimized conditions

Mycotoxin	Retention time [min]	Parent ion [m/z]	Product ions [m/z]	Collision energy	S-Lens
AFB1	6.34	313.1 $[M+H]^+$	241.1/285.1	37/22	129
AFB2	6.17	315.1 $[M+H]^+$	259.1/287.1	28/25	130
AFG1	6.06	329.0 $[M+H]^+$	243.0/199.1	25/60	61
AFG2	5.95	331.0 $[M+H]^+$	245.1/189.0	29/41	123
DON	5.40	297.1 $[M+H]^+$	249.1/231.1	11/13	63
OTA	10.21	404.1 $[M+H]^+$	239.0/221.0	23/34	94
ZEA	9.83	319.1 $[M+H]^+$	187.1/283.2	19/7	79
FB1	7.49	722.4 $[M+H]^+$	334.3/352.3	40/34	165
FB2	9.42	706.4 $[M+H]^+$	336.3/318.3	35/37	156
T-2	8.12	489.2 $[M+Na]^+$	245.1/327.1	25/22	114
HT-2	7.30	447.2 $[M+Na]^+$	285.1/345.1	15/17	102

3. Results and Discussion

3.1 *Method Validation*

Method validation was performed in terms of selectivity, linearity, accuracy, repeatability, inter-day precision, limits of detection and quantification (LOD, LOQ) and trueness of the method by analyzing CRMs and participating in FAPAS® proficiency tests.

Mycotoxins were quantified by using matrix-matched calibration curves in blank maize samples in order to have the same response between samples and calibration curve avoiding matrix effect. In this work, the method presents a single extraction prior to analysis without a clean-up procedure, which means that matrix components can affect the ionization of the target compounds, so this matrix effect was corrected by using the matrix-matched calibration curve to obtain reliable results. The identification of the compounds in the sample was positive when the retention time of the compounds in the sample had a tolerance of ±2.5% compared to that of the calibration standard injected in the same run and the ratio of the two ion transitions (quantification and confirmatory ion) of each compound was similar to the calibration standards with acceptance limits according to the EU Directive 96/23/EC.[11] Fig. 2 shows a typical SRM chromatogram of a spiked maize sample with 300 µg/kg of DON, 5 µg/kg of AFB2, AFG1 and AFB2, 4 µg/kg of AFB1 and OTA, 200 µg/kg of fumonisins, 250 µg/kg of ZEA and 41 µg/kg of toxins HT-2 and T-2.

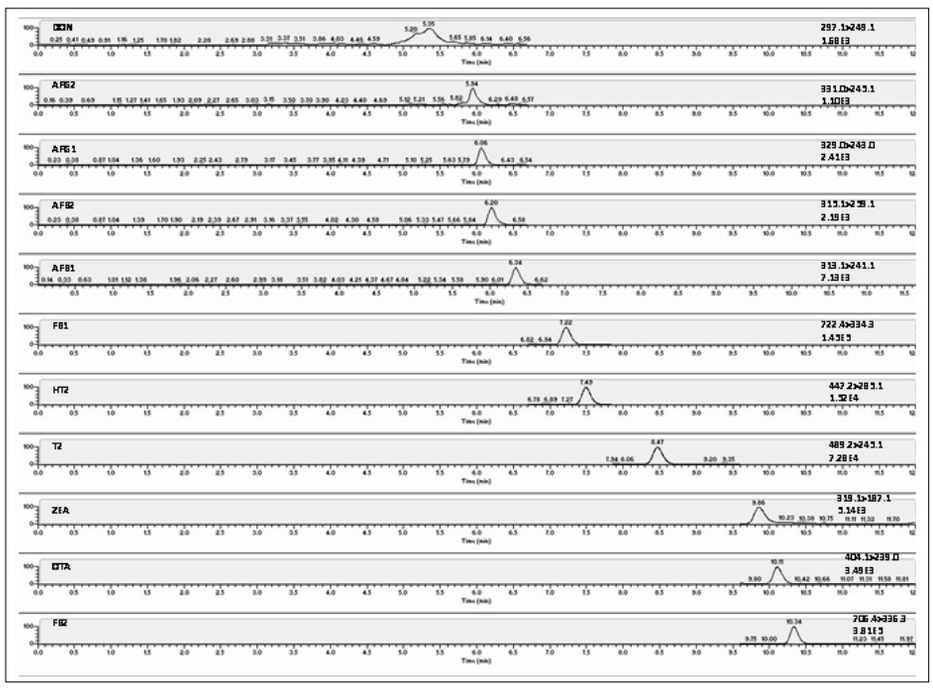

Fig. 2. LC/MS/MS chromatograms obtained from a blank maize sample spiked at 300 µg/kg of DON, 5 µg/kg of AFB2, AFG1, AFG2, 4 µg/kg of AFB1, OTA, 200 µg/kg of fumonisins, 250 µg/kg of ZEA and 41 µg/kg of toxins HT-2 and T-2.

The linearity of the method was tested by preparing eight replicates of spiked blank maize samples at six different concentrations between 100–350 µg/kg for DON, 0.5–5 µg/kg for AFB1 and OTA, 1–6 µg/kg for AFB2, AFG1 and AFG2, 25–250 µg/kg for FB1 and FB2, 5–50 µg/kg for T-2 and HT-2 and finally 50–300 µg/kg for ZEA. The choice of concentration ranges was a compromise in order to have EU and CH maximum levels (Table 2) within or below the calibration range, to meet linearity specifications and to have the lowest level close to the LOD (Table 2). Since no maximum levels exist for toxins T-2 and HT-2, the indicative level proposed by the EU of 100 µg/kg was taken into account. Samples with analyte concentrations above the calibration range were diluted and reanalyzed. Linear, 1/x weighted calibration curves were constructed from the data with correlation coefficients (R) in the range of 0.973–0.999.

Recoveries were calculated by the ratio of the measured to spiked concentrations. The recovery of each mycotoxin is determined by analyzing blank maize sample spiked before extraction at two different concentration levels of 100 µg/kg and 350 µg/kg for DON, 0.5 µg/kg and 5 µg/kg for AFB1 and OTA, 1 µg/kg and 6 µg/kg for the other AFs, 25 µg/kg and 250 µg/kg for FBs, 50 µg/kg and 300 µg/kg for ZEA and 5 µg/kg and 50 µg/kg for T-2 and HT-2. Recovery experiments were performed in 10 replicates (n=10) for each concentration. The recovery ranges were between 95.2%–115.3% which means that matrix-matched calibration curve can compensate matrix effects and fulfills the requirements established by Swiss legislation.[3]

Intra-day repeatability expressed as RSD (n=6) was between 4% and 13% and RSD (n=6) inter-day precision was between 3% and 20% for the low and the high spiked levels of the calibration range. Thus, the method fulfills Swiss criteria.[3]

Sensitivity was evaluated by LOD and LOQ values (Table 2). The LOD and LOQ were estimated from analysis of matrix-matched standards, and they were determined at the lowest concentration of the compounds that produce chromatographic peaks at S/N of 3 and 9, respectively. In the same table maximum levels recommended by the EU and Swiss legislation for maize

Table 2. LODs, LOQs for analysis of mycotoxins in maize matrix, EU, CH established maximum levels and cut-off values [µg/kg]

Mycotoxin	AFB1	AFB2	AFG1	AFG2	DON	OTA	ZEA	FB1	FB2	T-2	HT-2
LOD [µg/kg]	0.5	0.4	0.5	0.8	50	0.2	17	33	10	0.8	0.8
LOQ [µg/kg]	1.5	1.3	1.6	2.5	150	0.6	50	100	30	2.5	2.5
Maximum levels (EU, CH) [µg/kg][3,4]	5				750	3	100	1000 (sum of fumonisins)			
	4 (sum of aflatoxins)										
Cut off values [µg/kg]	4				600	2.4	80	800 (sum of fumonisins)		80 (sum of toxins T-2 and HT-2)	
	3.2 (sum of aflatoxins)										

are compared with the results obtained. For all regulated mycotoxins, LOQs are lower than the limits established.

The method was tested for trueness by analyzing CRM and FAPAS proficiency test materials. The accuracy of the concentrations obtained was controlled by calculating recovery and z-score taking into account the certified/assigned value. The Trilogy®CRM and the FAPAS 04201 were used to validated the method. For both analysis was repeated six times.

The trueness values obtained for all mycotoxins for the Trilogy®CRM were within 77–118% and all z-scores obtained were $-2 \leq z \leq 2$ except for FAPAS 04214 for AFB1 (z-score = –2.2), a result that demonstrates the suitability of the method as a semi-quantitative approach (Table 3). As it is shown, results can be underestimated so cut off values are set at 80% of the CH/EU maximum levels (Table 2). For the toxins T-2 and HT-2 no maximum levels have been established, so it was decided to set a cut off value at 80 µg/kg for the sum of these toxins (corresponding to the indicative level proposed by the EU: 100 µg/kg). In routine analysis, when a non compliance is present or the obtained values are at 80% of maximum levels, a quantitative analysis is performed by using a specific method with an immunoaffinity column clean-up before analysis for confirmation.

Table 3. Comparison of trueness data during analysis of one CRM and four FAPAS proficiency tests

Matrix	Analyte	Assigned value [µg/kg]	Results [µg/kg]	Trueness [%]	z-score
Trilogy®CRM	DON	2600.00	2011.00	77	n.a.
	AFB1	18.80	15.00	80	n.a.
	AFB2	0.90	n.d.		n.a
	AFG1	2.40	2.04	85	n.a
	AFG2	n.d.	n.d.	100	n.a.
	FB1	28300.00	24432.00	86	n.a.
	FB2	7100.00	8366.00	118	n.a.
	ZEA	352.00	404.00	115	n.a.
	OTA	4.00	3.00	75	n.a.
	T-2	263.70	269.00	102	n.a.
	HT-2	523.30	476.40	91	n.a.
FAPAS 04201	DON	2013.00	1506.00	75	–1.75
	AFB1	6.80	6.78	100	–0.01
	ZEA	396.00	395.70	100	0.00
	OTA	8.34	9.57	115	0.67
FAPAS 04214	DON	1329.00	920.00	69	–2.0
	AFB1	13.20	6.90	52	–2.2
	ZEA	290.00	284.00	98	–0.1
	OTA	8.89	9.90	111	0.5
FAPAS 04223	DON	1247.00	1321.40	106	0.4
	AFB1	5.42	5.53	102	0.1
	FB1	797.00	792.00	99	–0.03
	FB2	360.00	304.00	84	–0.61
	ZEA	286.00	340.50	119	1.0
	OTA	2.40	2.41	100	0.0
	T-2	160.00	118.00	74	–1.3
	HT-2	105.00	75.00	71	–1.3

3.2 *Application to Commercial Samples*

In total, 20 maize samples of 1 kg per sample were collected from retail outlets in Switzerland in the area of the canton of Vaud and analyzed in order to investigate the presence of the 11 mycotoxins. Prior to analysis these samples were milled to a fine flour and homogenized in the same way as the blank sample for the calibration curve.

In every sequence of analysis, a blank maize sample, a reagent blank and a spiked blank used to evaluate the reliability of the proposed method are added. The spiked concentrations were at the limit values according to the Swiss legislation[3] for the mycotoxins whose limit values were in the calibration curve's range, otherwise for T-2 and HT-2, the spiked concentrations corresponded to the middle concentration of the calibration curve. In the positive samples, each mycotoxin was identified by choosing the appropriate retention time window and confirmation was performed by comparison of the signal intensity ratios of the two transitions, quantification and verification, to the two transitions obtained from the spiked blank samples according to tolerances fixed by the EU.

In Table 4 the results of the samples analyzed are presented. In these maize samples, aflatoxins, ochratoxin A and zeara-

Table 4. Distribution of mycotoxins in twenty maize samples

Mycotoxin	Mycotoxin content [µg/kg]																			
	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	S 10	S 11	S 12	S 13	S 14	S 15	S 16	S 17	S 18	S 19	S 20
AFB1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Σ AFs	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
DON	–	–	–	126	–	–	–	125	–	–	–	–	479	548	311	–	–	–	340	300
Σ FBs	–	430	210	99	21	23	17	33	–	19	625	32	109	19	18	80	42	24	41	–
OTA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ZEA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Σ(T-2,HT-2)	–	1.6	0.62	5	19	4	–	–	–	–	–	–	–	–	–	–	–	3	3	5

lenone were not detected. Fumonisins B1 and B2 were present in 85% of the samples with one sample at the highest level of 625 µg/kg, three samples showed a medium content (430, 210 and 109 µg/kg) and thirteen samples showed a low content. Toxins T-2 and HT-2 were present in 40% (eight samples), all samples had a low content and DON was present in 35% (in seven samples). The content of DON was found in two samples at a high level (479 and 548 µg/kg), in three of them at a medium level (300–340 µg/kg) and two of them showed a low level (125and 126 µg/kg). All values are below the maximum limits established by Swiss legislation[3] and the cut off values set up, so no confirmatory method was used. The developed analytical method was shown to be effective for identifying mycotoxins in samples as well as providing a semi-quantitative tool for the positive samples by using a fast and easy extraction method.

4. Conclusion

The multi-mycotoxin method was developed for the simultaneous determination of 11 mycotoxins in a maize matrix. This technique provides an easy and rapid screening tool for maize samples. Due to the high sensitivity of the triple quadrupole, a single extraction is sufficient to reach low detection limits, fulfilling Swiss law requirements. Finally, the validated method was used to analyze commercial maize samples which allows 11 mycotoxins to be monitored simultaneously and so enable a quicker assessment of mycotoxin contamination. Further research is planned to study the potential of this method to analyze other cereal complex matrices such as wheat, barley, rice, breakfast cereals, and cereal-based baby food and so determine its general applicability.

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