Application Note

Food Testing

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Quantification of Mycotoxins in Food Matrices

2D-Liquid Chromatography with the Agilent 6495D Triple Quadrupole LC/MS System

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Abstract

This application note demonstrates the use of two-dimensional liquid chromatography (2D-LC) with mass spectrometry (MS) for the analysis of mycotoxins in food matrices. The mycotoxins aflatoxin B1, B2, G1 and G2, as well as ochratoxin A were analyzed in pasta, brown rice, and cayenne pepper matrices. 2D-LC enables measurement of sample extracts directly after a simple solid/liquid extraction, eliminating the need for time-consuming purification and enrichment steps such as solid-phase extraction (SPE) or immunoaffinity chromatography (IAC). In addition, the method achieves a total injection runtime of just 14 minutes across both dimensions and operates on a single software platform for the entire 2D-LC workflow, streamlining implementation and control. To demonstrate the benefits of 2D-LC, extracts of blank matrices were spiked and analyzed. The low-level spike (0.08 ng/mL) fulfilled the legally required limit of quantitation (LOQ) for all matrices and mycotoxins with excellent measurement precision (0.71–10.28% area RSD) and recovery (70–120%).

Introduction

Mycotoxins are secondary metabolites formed by fungi that can enter the food chain in various ways, for example when a crop is infested in the field or during storage after harvest. Grain, seeds, dry fruits, nuts, and their processed products are particularly affected. At certain levels, mycotoxins can have serious effects on health, including carcinogenic, mutagenic, and neurotoxic effects. To protect consumers, the European Union (EU) has issued maximum residue levels (MRLs) for specific mycotoxins including aflatoxins (B1, B2, G1, and G2) and ochratoxin A (OTA), in various foods.[1]

Mycotoxins are usually analyzed using high-performance liquid chromatography (HPLC) with fluorescence detection (FLD) or HPLC/MS/MS. However, sample preparation can be time-consuming and costly if the sample has to be purified and the analytes enriched by solid phase extraction (SPE) or immunoaffinity chromatography (IAC). In addition, the method used must balance effective matrix removal, high recovery, and adequate reproducibility.[2,3]

An alternative approach is the use of two-dimensional liquid chromatography coupled to triple quadrupole mass spectrometry (2D-LC/TQ) using a multiple heart-cutting. In a multiple heart-cutting experiment, time-dependent aliquots (cuts) from selected peaks from the first separation column (first dimension or 1D) are collected and temporarily stored in dedicated loops. These aliquots are then transferred to the second dimension, where coeluting compounds are further separated using different stationary phase-eluent combinations. As a result, matrix effects and interference are reduced, making direct analysis of mycotoxins in complex extracts possible.[4]

This application note demonstrates the benefits of 2D-LC/TQ for the analysis of mycotoxins in food matrices. Agilent MassHunter Acquisition software completes the solution, enabling easy setup and control of the 2D-LC/MS system in a single software platform[5] (Figure 1).

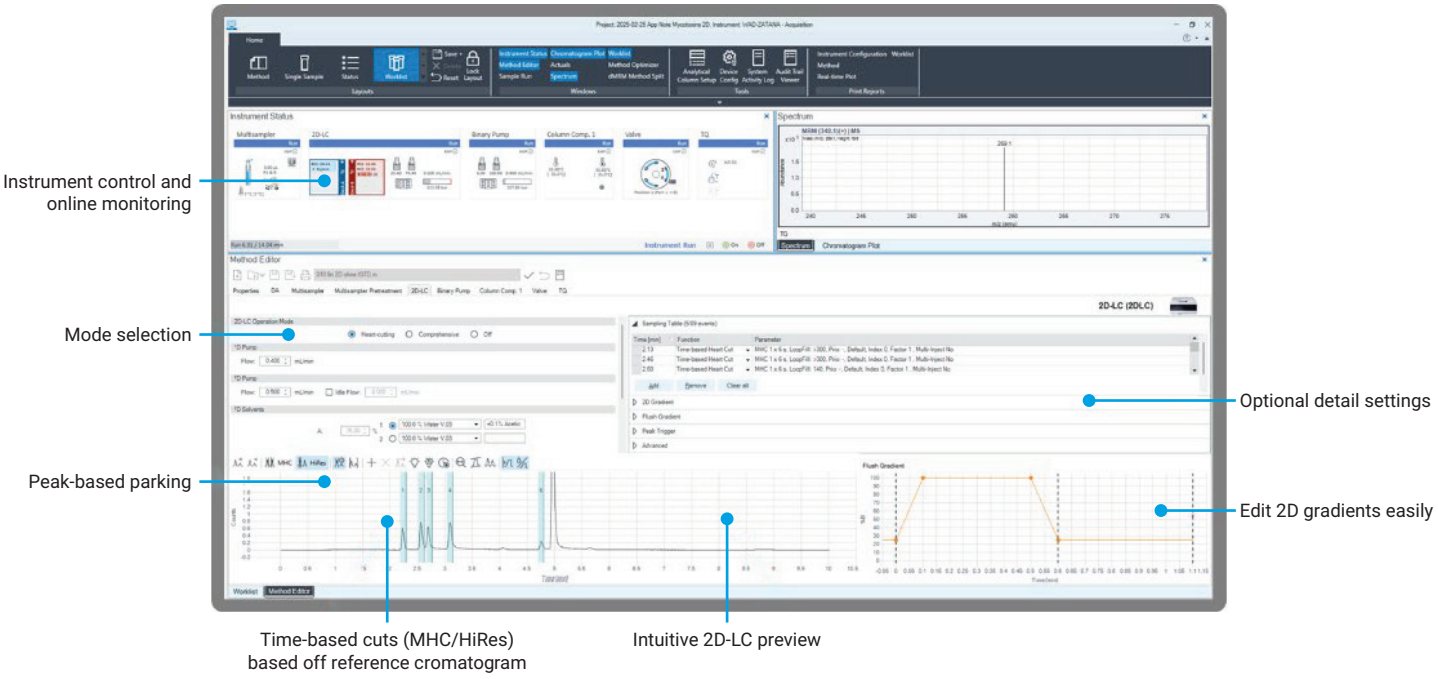

Figure 1. Agilent MassHunter Acquisition software revision 12 for 2D-LC methods.

Experimental

Chemicals and reagents

All reagents and solvents were MS or analytical grade. Methanol, acetonitrile, and acetic acid were purchased from VWR. Ammonium acetate was from Sigma-Aldrich. The mycotoxins and internal standards (see Table 5) were obtained from Biopure (Romer labs).

Sample preparation

Five grams of a well-homogenized sample were extracted with a mixture of acetonitrile/water. The suspension was homogenized by shaking and separated via centrifugation. An aliquot of the liquid phase was diluted with an aqueous solution to yield a final measurement extract with an organic-to-water ratio of 26/74 (v/v).

Preparation of calibration levels and matrix spikes

To prepare the calibration levels, a standard target solution was prepared in a 20/80 (v/v) mixture of acetonitrile and water at a concentration of 50 ng/mL for all compounds and further diluted. Ten calibration levels were prepared to cover the range from 0.01 to 5 ng/mL.

The internal standard was added to the calibration levels and samples prior to injection using an injector program on an Agilent 1290 Infinity II System Multisampler. For this purpose, a standard solution was prepared, which contained isotope-labeled internal standards in the following concentrations: $^{13}C_7$-Aflatoxin B1 (2.52 ng/mL), $^{13}C_7$-Aflatoxin B2 (2.54 ng/mL), $^{13}C_{17}$-Aflatoxin G1 (2.50 ng/mL), $^{13}C_{17}$-Aflatoxin G2 (2.53 ng/mL), and $^{13}C_{20}$-OTA (5.98 ng/mL).

The developed method was tested for different food matrices by spiking extracts of blank matrices. A high- and low-level matrix spike was prepared. For the high-level spike, a 15 ng/mL standard was prepared, and 20 µL of the standard target solution was added to 130 µL of a matrix extract, resulting in a final concentration of 2 ng/mL (24.01 µg/kg). For the low-level spike, a 1 ng/mL standard solution was prepared and 16 µL of the standard was added to 184 µL of matrix extract, resulting in a final concentration of 0.08 ng/mL (0.96 µg/kg). The low-level matrix spike represents the required LOQ per Commission Implementing Regulation EU 2023/2782[6] (Table 6).

Instrumentation

The Agilent 1290 Infinity II 2D-LC Solution was configured with:

- Two Agilent 1290 Infinity II High Speed Pumps (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with cooler (#101)
- Two Agilent 1290 Infinity II Multicolumn Thermostats (G7116B)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2-position/6-port valve (G4231A) (1D/2D-Selection valve)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2D-LC Active Solvent Modulation (ASM) Valve (G4243A)
- Two Agilent 1290 Infinity Valve Drives (G1170A) with Multiple Heart-Cutting Valves (G4243A, #007, #008) equipped with 40 µL loops

Because the Agilent 1290 Infinity III LC System and its accessories provides similar performance, they can be applied to this method to obtain similar results.

Mass selective detection was performed using an Agilent 6495D Triple Quadrupole (TQ) LC/MS equipped with an Agilent Jet Stream electrospray ionization (ESI) source.

Method

The LC conditions for the first and second dimension separations are in Tables 1 and 2. The analytes in the first dimension were eluted in 8.1 minutes using an Agilent Poroshell Phenyl Hexyl column (part number 699775-912). Afterwards, the column was equilibrated with the starting conditions of the first dimension, while the measurements in the second dimension were carried out on an Agilent ZORBAX Eclipse Plus C18 column (part number 959757-902). Since the goal was to analyze five mycotoxins, five cuts were performed to temporarily store the analytes in dedicated loops, which were then successively transferred to the second dimension and analyzed on the 2D column with the analytical gradient in Table 3. The runtime including backwash and equilibration in the second dimension was 1.75 minutes per cut. The total runtime per injection, including the first and second dimension, was 14 minutes.

The internal standard was added using an Agilent 1290 Infinity II LC System Multisampler injector program that also defined autosampler needle cleaning (Table 4).

Table 1. Agilent 1290 Infinity II LC System method for the first and second dimension separations.

Parameter	1D-LC Method	2D-LC Method
Sample Temperature	6 °C	–
Injection Volume	3 µL	–
Column	Agilent Poroshell Phenyl Hexyl, 2.1 × 50 mm, 2.7 µm (p/n 699775-912)	Agilent ZORBAX Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm (p/n 959757-902)
Column Temperature	35 °C	35 °C
Flow Rate	400 µL/min	500 µL/min
Mobile Phase A	2 mM ammonium acetate + 0.1 % acetic acid	2 mM ammonium acetate + 0.1 % acetic acid
Mobile Phase B	Methanol	Methanol:acetonitrile (50:50; v:v)
Gradient Program	Time (min) %B 0 5 0.50 45 4.00 60 5.00 100 8.00 100 8.10 5 Holding starting conditions until measurements in the second dimension are completed.	Analytical gradient: Time (min) %B 0 25 0.05 60 1.20 80 1.25 25 1.75 25 Flush gradient: Time (min) %B 0 25 0.10 100 0.50 100 0.60 25

Table 2. 2D-LC conditions.

Parameter	Value
Configuration	2D-LC valve ASM + 2 MHC valves, 40 µL loops
2D-LC Mode	Multiple heart-cutting
Active Solvent Modulation (ASM)	ASM capillary: 5500-1301 (0.12 × 170 mm) ASM enabled (ASM factor 3.0) Flush sample loop: 0.7 times
Sampling Table	2.12 min, time-based 2.45 min, time-based 2.58 min, time-based 2.98 min, time-based 4.65 min, time-based

Table 4. Agilent 6495D LC/TQ parameters.

Parameter	Value
Ion Source	Agilent Jet Stream ESI Source
Polarity	Positive
Gas Temperature	240 °C
Drying Gas Flow	17 L/min
Nebulizer	40 psi
Sheath Gas Temperature	380 °C
Sheath Gas Flow	12
Capillary Voltage	3,000 V
Nozzle Voltage	0 V
Scan Type	Multiple reaction monitoring (time segments)
Detector Gain Factor (+)	3

Table 3. Agilent 1290 Infinity II LC System Multisampler program.

Parameter	Value				
Injector Program	1. Draw 3 µL of sample 2. Wash needle as defined in method (multiwash) 3. Draw 1.50 µL of internal standard 4. Wash needle in flushport with ACN:isopropanol:THF (1:1:1) for 12 s 5. Inject				
Multiwash	Step	Solvent	Time (s)	Seat backflush	Needle wash
	1	ACN:Iso:THF (1:1:1)	15	Yes	Yes
	2	Methanol	15	Yes	Yes
	3	Water	15	Yes	Yes
	Starting conditions	Water		Yes	No

MS data were acquired in positive ion mode with a constant fragmentor setting of 166 V and a dwell time of 30 ms for each ion transition. The ion source and mass spectrometer parameters are in Table 4, and the 6495D LC/TQ multiple reaction monitoring (MRM) parameters per compound are in Table 5. The iFunnel mode optimizes mass spectrometry performance by dynamically adjusting the radiofrequency (RF) amplitude for each compound, with predefined settings for Fragile, Standard, and Large Molecules. MassHunter Acquisition software revision 12.1 and 2D-LC software for MassHunter (G2198AA) were used for data acquisition. Data were processed using MassHunter Quantitative analysis for QQQ software revision 12.1.

Table 5. Mycotoxins and internal standards, and Agilent 6495D LC/TQ multiple reaction monitoring (MRM) parameters per compound.

Compound Name	Precursor *m/z*	MS1 Resolution	Product *m/z*	MS2 Resolution	CE (V)	CAV (V)	iFunnel Mode
Aflatoxin B1	313.0	Unit	241.0	Wide	44	5	Standard
	313.0	Unit	285.0	Wide	24	5	Standard
Aflatoxin B2	315.2	Unit	259.1	Wide	30	5	Standard
	315.2	Unit	287.1	Wide	24	5	Standard
Aflatoxin G1	329.2	Unit	200.1	Wide	41	4	Standard
	329.2	Unit	214.1	Wide	40	4	Large Molecule
	329.2	Unit	243.1	Wide	25	5	Standard
	329.2	Unit	311.1	Wide	20	5	Standard
Aflatoxin G2	331.2	Unit	115.0	Wide	80	4	Standard
	331.2	Unit	245.1	Wide	30	5	Large Molecule
	331.1	Unit	313.1	Wide	23	5	Large Molecule
OTA	404.1	Unit	221.0	Wide	36	5	Standard
	404.1	Unit	239.0	Wide	24	5	Standard
	404.1	Unit	358.0	Wide	13	5	Standard
$^{13}C_7$-Aflatoxin B1	330.1	Unit	301.1	Wide	21	5	Standard
	330.1	Unit	255.1	Wide	44	5	Standard
$^{13}C_7$-Aflatoxin B2	332.2	Unit	303.0	Wide	21	4	Standard
	332.2	Unit	272.8	Wide	30	4	Standard
$^{13}C_{17}$-Aflatoxin G1	346.1	Unit	212.2	Wide	41	5	Standard
	346.1	Unit	257.1	Wide	25	4	Standard
$^{13}C_{17}$-Aflatoxin G2	348.1	Unit	330.1	Wide	23	4	Large Molecule
	348.1	Unit	124.0	Wide	80	4	Standard
$^{13}C_{20}$-OTA	424.2	Unit	250.1	Wide	25	5	Standard
	424.2	Unit	232.1	Wide	36	5	Standard

Results and discussion

Linearity and measurement precision

A linear regression with a weighting of 1/x was used to evaluate linearity. The aflatoxins B1, B2, and G1 showed good linearity from 0.01 to 5 ng/mL. For aflatoxin G2 and OTA, the linear range started at 0.03 ng/mL. The correlation coefficient (R^2) for all analytes was greater than 0.998 (Figure 2). Furthermore, excellent precision was achieved for the multiple heart-cutting method. For seven replicate injections of the 0.08 ng/mL calibration level (required LOQ), peak area and retention time RSDs were between 1.89 and 4.53%, and ≤ 0.07%, respectively. These results demonstrate the robustness of the method and the cut settings used. The chromatograms at the 0.08 ng/mL calibration level are shown in Figure 3A.

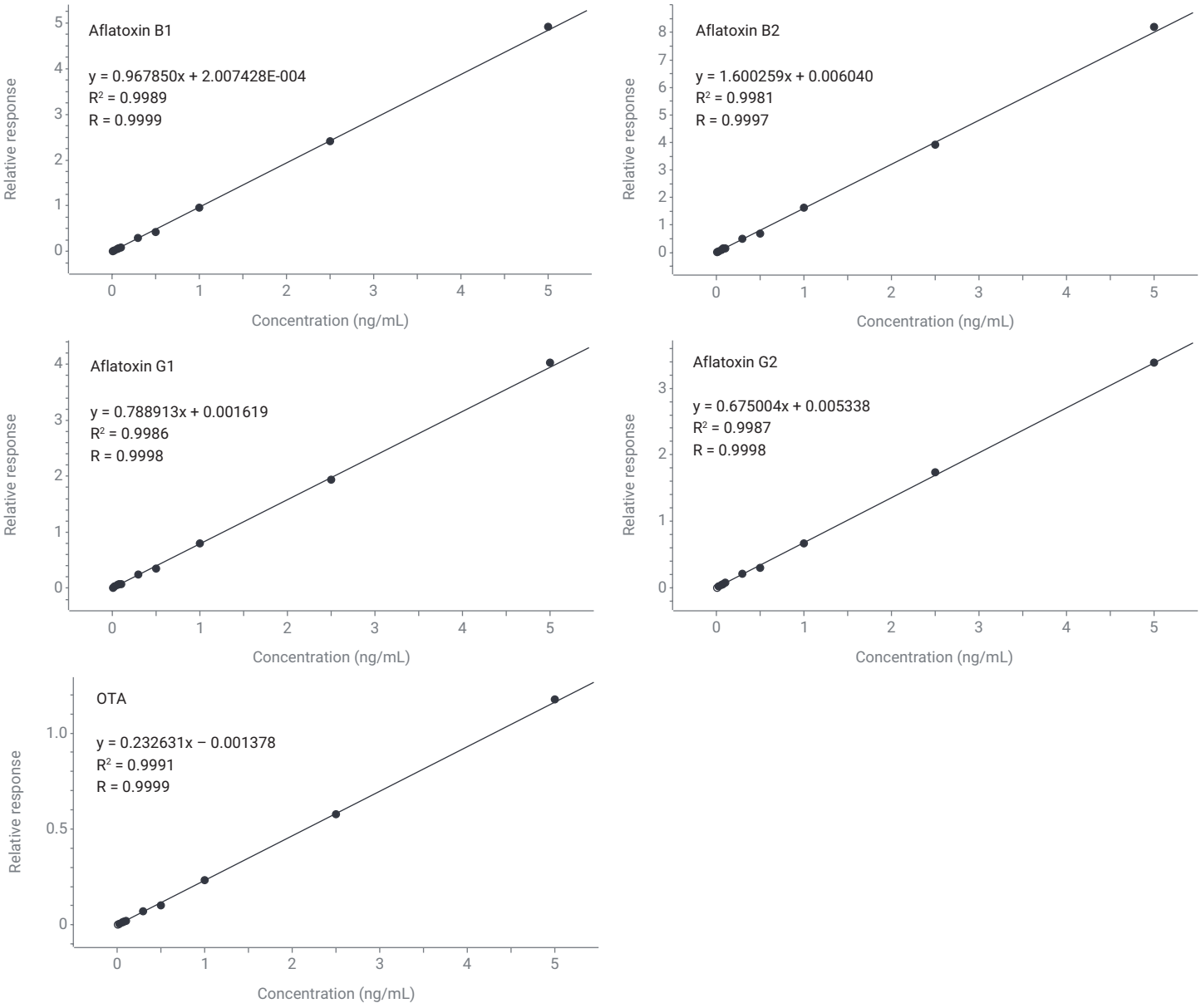

Figure 2. Calibration curves for aflatoxins B1, B2, G1, and G2, and ochratoxin A.

Measurement recovery and reproducibility

To demonstrate the potential of 2D-LC for mycotoxin analysis, the recovery in the different food matrices was tested by measuring a low- (0.08 ng/mL ≙ 0.96 µg/kg) and a high-level spike (2 ng/mL ≙ 24.01 µg/kg). The concentration of the low-level spike, in relation to the weight of the matrix, covered the required LOQ for the individual mycotoxins per Commission Implementing Regulation EU 2023/2782[6] as shown in Table 6. Sample chromatograms for the low-level spikes are shown in Figures 3B, 3C, and 3D. The measurement recovery was between 70–120% for all analytes and matrices with the internal standard factored into the calculation (Figure 4). The RSDs of the concentrations of the low-level spikes of three injection replicates were 0.71–8.18%.

Table 6. Current maximum residue level (MRL) for mycotoxins in various foods (EU 2023/915) and required LOQ per Commission Implementing Regulation (EU 2023/2782).

Mycotoxin	MRL (µg/kg)			Required LOQ (µg/kg)
	Cayenne Pepper	Brown Rice	Pasta	
Aflatoxin B1	5.0	2.0	2.0	≤ 1.0 for each aflatoxin
Aflatoxin B2	∑ 10.0	∑ 4.0	∑ 4.0	
Aflatoxin G1				
Aflatoxin G2				
Ochratoxin A	20.0	3.0	3.0	10.0 (Cayenne pepper) 1.5 (Brown rice, pasta)

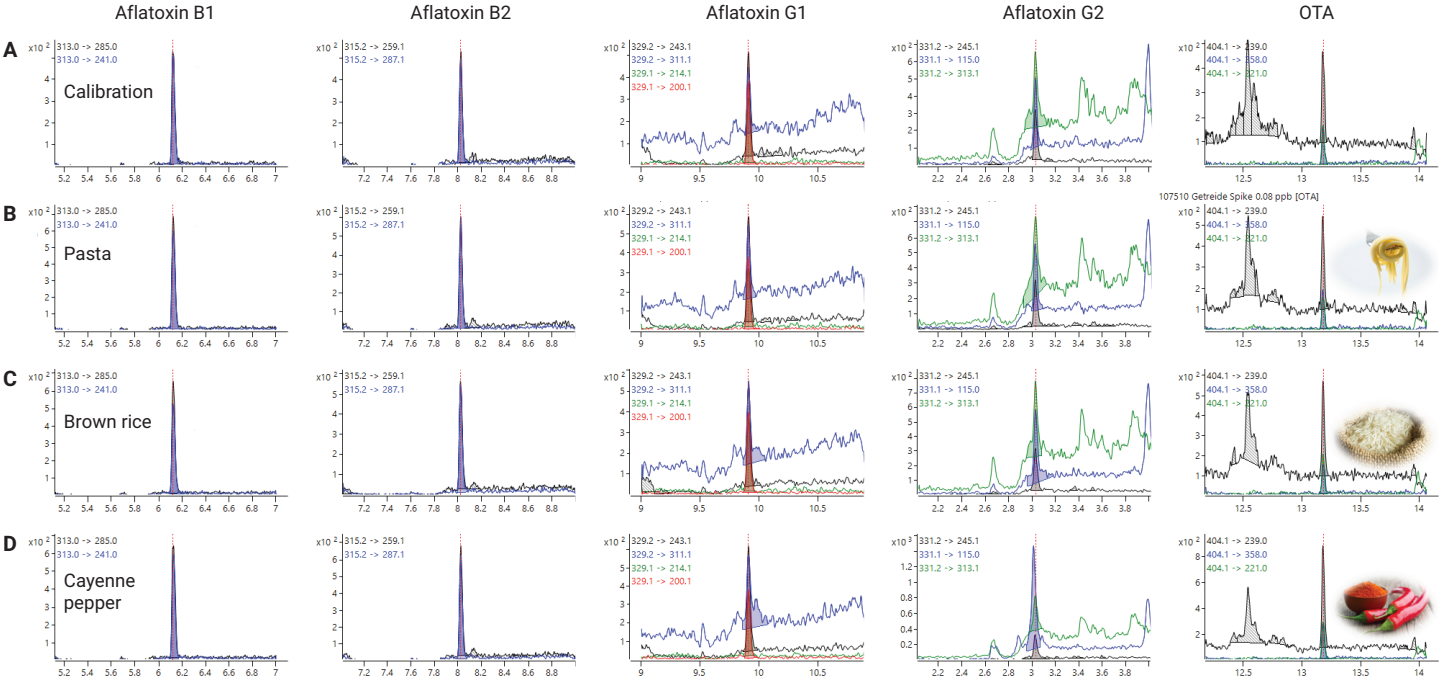

Figure 3. Chromatograms of the mycotoxins analyzed at the 0.08 ng/mL calibration level (A) and the low-level spikes (0.08 ng/mL) of the extracts of the blank matrices, (B) pasta, (C) brown rice, and (D) cayenne pepper.

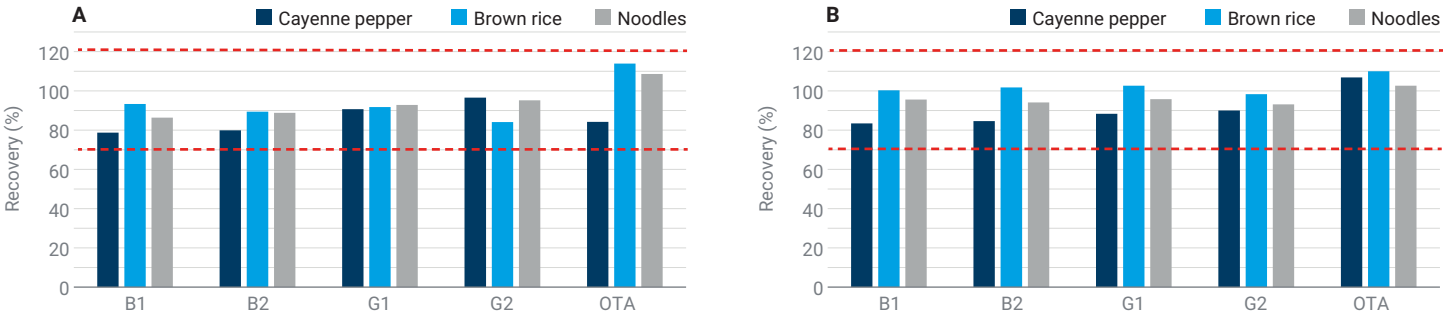

Figure 4. Measurement recoveries of the (A) low- (0.08 ng/mL, n = 3) and (B) high-level spike (2 ng/mL, n = 3) for the different food matrices analyzed.

Conclusion

This application note highlights the performance of 2D-LC for the determination of mycotoxins in food matrices, which offers a more efficient approach than methods that require time-consuming and cost-intensive SPE or IAC sample preparation. 2D-LC extends the chromatographic separation into an additional dimension to reduce matrix effects and enable direct measurement of sample extracts, making time-consuming and costly purification and sample enrichment by SPE or IAC unnecessary. The presented method showed good measurement recovery and precision. Analysis of spiked extracts of blank matrices of different foods showed that the legally required LOQ[1,6] can be achieved. Agilent 2D-LC software for Agilent MassHunter Workstation software provides an easy-to-use, single-software-platform solution for easy setup and control of a 2D-LC/MS system comprised of an Agilent 1290 Infinity II LC System coupled to an Agilent 6495D LC/TQ.

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