Determination of Multiple Mycotoxins in Grain Using a QuEChERS Sample Preparation Approach and LC-MS/MS Detection

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Key Words
Mycotoxins, food, HyperSep, QuEChERS, dispersive SPE, Accucore aQ, TSQ Vantage

Goal

To demonstrate a fast, easy, and cost-effective approach for the determination of 16 mycotoxin residues in grain-based food using QuEChERS sample preparation with a Thermo Scientific™ Accucore™ aQ HPLC column and a Thermo Scientific™ TSQ™ Vantage™ triple quadrupole mass spectrometer for HPLC separation.

Introduction

Mycotoxins are toxic secondary metabolites that are naturally produced by several species of fungi on agricultural products, particularly grain-based products. Mycotoxins are chemically stable and cannot be destroyed during food processing and heat treatment. Therefore, they may occur in the field, in raw materials during storage, and in processed foods. To date, more than 300 mycotoxins, possessing varying degrees of toxicity, have been identified, although only a relatively few of these are widely accepted as presenting a significant food or animal feed safety risk.[1] Studies have shown that mycotoxins can have significant adverse effects on human health, even at very low concentrations.[2] Therefore, monitoring and inspecting the contamination levels of mycotoxins in foods and feeds has gained major national and international attention over the years.

The analysis of mycotoxins is challenging due to the large number of compounds to be detected and the wide physicochemical properties they possess. In addition, the foods tested are typically complex in nature and may be simultaneously contaminated with several mycotoxins at low concentrations. Sample preparation approaches that have been reported for mycotoxin analysis include solid-supported liquid-liquid extraction (SLE), liquid–liquid extraction, matrix solid-phase dispersion, immunoaffinity chromatography, solid-phase extraction and QuEChERS. All approaches are complicated by the considerably different polarity and solubility of the mycotoxins, in particular the polar trichothecenes, and finding a solvent system that will simultaneously extract all the mycotoxins with good efficiency is difficult. Therefore, due to the fact that sample cleanup is often limited, final extracts may still contain large amounts of matrix components that can negatively affect the detection system.

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has become the most widely used detection system for mycotoxin analysis. LC-MS/MS offers the advantage of good selectivity and sensitivity, and it has the ability to cover a wide range of compounds. Difficulties encountered in LC-MS/MS analysis of mycotoxins include finding conditions suitable for all the mycotoxins, obtaining adequate LC retention and peak shape for the polar trichothecenes, and overcoming matrix effects. The latter issue is particularly relevant to trichothecenes as their ionization efficiency can be affected by the presence of co-eluting matrix interferences, leading to signal suppression or enhancement.[3] Some mycotoxins, such as the aflatoxins or α- & β-zearalanol, are structurally similar, and their analysis requires high

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chromatographic selectivity and resolution. The method described in this application note uses an Accucore aQ HPLC column for the fast and efficient chromatographic determination of 16 mycotoxins and 3 internal standards.

Accucore HPLC columns use Core Enhanced Technology™ to facilitate fast and high-efficiency separations. The 2.6 µm diameter particles have a solid core and a porous outer layer. The optimized phase bonding creates a series of high-coverage, robust phases. The tightly controlled 2.6 µm diameter of Accucore particles results in much lower backpressures than typically seen with sub-2 µm materials. Accucore aQ columns are compatible with 100% aqueous mobile phases and offer enhanced retention and selectivity for polar analytes.

This application note outlines a fast, simple, and efficient QuEChERS method for the routine analysis of 16 mycotoxins in grain-based food. The compounds included in this method are representative of a wide range of mycotoxins, including type A- and B-trichothecenes, ochratoxin A, alternariol, zearalenone, α- & β-zearalanol , and aflatoxins (B1, B2, G1, G2). Detection and quantitation is carried out using an Accucore aQ HPLC column coupled to a TSQ Vantage triple quadrupole mass spectrometer.

Experimental

Chemicals and Reagents

- Fisher Scientific™ acetonitrile (HPLC grade)
- Fisher Scientific methanol (HPLC grade)
- Fisher Scientific formic acid (ACS grade), (P/N A118P-500)
- Fisher Scientific ammonium formate (LC-MS grade), (P/N A115-50)
- Fisher Scientific water (HPLC grade) or ultrapure water (18.2 mΩ)

Sample Preparation Supplies

- Fisherbrand™ Higher-Speed Easy Reader™ Plastic Centrifuge Tubes, 50 mL (P/N 06-443-18)
- Fisherbrand Disposable Heavy-Wall Borosilicate Glass Tubes, 12 × 75 mm (P/N 14-958-10B)
- Thermo Scientific™ National™ Target™ All-Plastic Disposable Syringes, luer-slip, 1 mL capacity (P/N S7510-1)
- Thermo Scientific™ Target2™ Nylon Syringe Filters, 0.2 µm, 17 mm (P/N F2513-2)
- Thermo Scientific borosilicate glass vials (2 mL, 12 mm × 32 mm) with 8 mm black screw cap fitted with a silicone/PTFE seal (P/N 60180-600)
- Thermo Scientific™ HyperSep™ Dispersive SPE Mylar™ Pouches, 4 g anhydrous $MgSO_4$ and 1g NaCl (P/N 60105-340)
- HyperSep Dispersive SPE Clean-up Product, 150 mg anhydrous $MgSO_4$, 50 mg PSA and 50 mg C18, 2mL microcentrifuge tube (P/N 60105-204)

Analytical Standards

Mycotoxin standards, of the highest possible purity, were purchased from reputable suppliers and supplied in either a solid form or as a pre-prepared solution. Individual solutions of the solid standards were prepared using appropriate solvent solutions. A working standard solution containing all 16 mycotoxins at a concentration of 1 µg/mL was prepared by mixing appropriate volumes of the stock solutions in a 10 mL volumetric flask and diluting to volume with methanol.

3-Acetyldeoxynivalenol-D_3, thiabendazole-$^{13}C6$, and gemfibrozil-D_6 were used as internal standards (IS). IS standard solutions were purchased from a reputable supplier and supplied in acetonitrile at a concentration of 100 µg/mL.

A 1 µg/mL working IS solution was prepared by mixing 100 µL of the 100 µg/mL stock solutions in a 10 mL volumetric flask and diluting to volume with methanol.

All stock standards and working solutions were transferred to amber glass vials with Teflon-lined caps and stored at -20 °C until needed.

Sample Preparation

Sample extraction

1. Place 5 g of sample into a 50 mL centrifuge tube.
2. Add 10 mL water.
3. Vortex briefly and allow to hydrate for at least 15 minutes.
4. Add 250 µL of 1 µg/mL internal standard. Add 10 mL acetonitrile containing 2% formic acid.
5. Shake/vortex samples for 15 minutes to extract mycotoxins.
6. Add the contents of a HyperSep Dispersive SPE Mylar Pouch (P/N 60105-340) containing the extraction salts.
7. Immediately shake for 1 minute.
8. Centrifuge for 5 min at ≥ 3000 *g*.

Sample cleanup

1. Transfer 1 mL of supernatant to a dSPE tube (HyperSep Dispersive SPE Clean-up Product P/N 60105-204).
2. Vortex for 30 seconds.
3. Centrifuge for 5 min at ≥ 3000 *g*.
4. Transfer 500 µL of purified supernatant to a 5 mL test tube.
5. Evaporate the acetonitrile extract to dryness and reconstitute with 500 µL methanol / water (50:50, v/v) for better chromatographic performance.
6. Filter the extract, using a 0.2 µm syringe filter (P/N F2513-2), directly into an autosampler vial (P/N 60180-600).

For improved sensitivity at low ppb concentrations, the dSPE step can be scaled-up by following the steps below:

1. Transfer 8 mL of supernatant to a 15 mL HyperSep Dispersive SPE Clean-up Product (1200 mg anhydrous $MgSO_4$, 400 mg PSA, and 400 mg C18, 15 mL centrifuge tube, P/N 60105-204).
2. Vortex for 30 seconds.
3. Centrifuge for 5 minutes at ≥ 3000 *g*.
4. Transfer 5 mL of supernatant to a 5 mL test tube.
5. Evaporate the sample to dryness at 40–50 °C under a gentle stream of nitrogen.
6. Reconstitute sample in 1 mL methanol / water (50:50, v/v).

Preparation of Matrix-Matched Calibration Curve

A five-point matrix-matched calibration curve was prepared using the sample extracts obtained from negative samples prepared according to the procedure described above but without the addition of standard or IS. Five 1 mL aliquots of blank extract were spiked with 5, 12.5, 25, 50, and 100 µL of the 1 µg/mL working standard, yielding a 5–100 ng/mL calibration range for each of the mycotoxins, which is equivalent to 10–200 µg/kg in samples.

Separation Conditions

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000
Column	Accucore aQ, 100 × 2.1 mm, 2.6 µm (P/N 17326-102130)
Guard column	Accucore aQ, 10 × 2.1 mm, 2.6 µm (P/N 17326-012105)
Run time	17 min (including 4 min equilibration)
Temperature	45 °C
Injection volume	5 µL
Autosampler temperature	10 °C
Wash solvent	Methanol / water (1:1, v/v)
Flow rate	400 µL/min
Waste divert	Mobile phase was diverted to waste from 0–1.5 and 13–17 min to reduce ion source contamination
Mobile phase A	10 mM ammonium formate in water
Mobile phase B	Methanol

Table 1. LC gradient.

Time (min)	A (%)	B (%)
0.0	100	0
1.0	75	25
4.0	75	25
5.0	60	40
8.0	60	40
8.5	40	60
9.5	40	60
10.0	0	100
13.0	0	100
13.2	100	0
17.0	100	0

MS Conditions

Instrumentation	TSQ Vantage tandem mass spectrometer
Ionization mode	$APCI^+$ & $APCI^-$ with fast polarity switching
Spray voltage	5 µA ($APCI^+$), 20 µA ($APCI^-$)
Vaporizer temperature	250 °C
Capillary temperature	250 °C
Sheath gas pressure	50 arbitrary units
Auxiliary gas pressure	15 arbitrary units
Ion sweep gas	0 arbitrary units
Declustering potential	0 V
Q1 and Q3 peak width	0.2 and 0.7 Da
Collision gas	Argon
Collision gas pressure	1.1 mTorr
Acquisition method type	EZ method (SRM)
Cycle time	0.4 s
SRM transitions are listed in Table 2.	

Data Processing

Thermo Scientific™ Xcalibur™ software version 2.2 was used. The weighting factor applied to the calibration curves was 1/X.

Table 2. SRM transitions.

Analyte	t_R (min)	Precursor ion		Product ion 2	CE 1	Product ion 2	CE 2	S-lens (V)
Nivalenol	2.47	357.34	$[M+HCOO]^-$	281.91	16	311.79	15	65
Deoxynivalenol	2.93	341.42	$[M+HCOO]^-$	265.87	13	295.96	16	63
Fusarenon X	3.56	354.90	$[M+H]^+$	136.97	31	174.96	19	76
Neosolaniol	3.96	399.91	$[M+NH_4]^+$	184.99	20	215.03	16	81
3-Acetyldeoxynivalenol	4.68	338.89	$[M+H]^+$	231.10	13	90.98	48	74
3-Acetyldeoxynivalenol-D_3	4.64	341.92	$[M+H]^+$	230.99	14	213.04	15	80
Aflatoxin G2	6.62	330.83	$[M+H]^+$	189.02	36	245.05	27	137
Aflatoxin G1	7.03	328.83	$[M+H]^+$	199.02	41	200.03	36	143
Thiabendazole-$^{13}C_6$	7.01	207.91	$[M+H]^+$	181.02	25	137.04	32	123
Aflatoxin B2	7.47	314.85	$[M+H]^+$	287.06	23	259.01	27	129
Aflatoxin B1	7.93	312.84	$[M+H]^+$	241.02	36	285.05	22	121
Diacetoxyscirpenol	8.10	383.93	$[M+NH_4]^+$	247.06	13	229.08	16	82
Ochratoxin A	9.18	403.80	$[M+H]^+$	238.93	23	220.90	36	101
Alternariol	10.08	257.65	$[M-H]^-$	214.03	23	216.01	26	113
β-zearalanol	10.48	321.51	$[M-H]^-$	277.94	24	303.86	24	125
α-zearalanol	11.11	321.51	$[M-H]^-$	277.94	24	303.86	24	125
T-2 toxin	11.09	483.91	$[M+NH_4]^+$	185.02	21	214.99	17	84
Zearalenone	11.30	317.50	$[M-H]^-$	176.13	27	273.95	22	110
Gemfibrozil-D_6	11.66	255.79	$[M-H]^-$	122.41	21	–	–	60

Results and Discussion

Liquid Chromatography-Mass Spectrometry

Developing an LC-MS/MS method for the analysis of multiple mycotoxins is challenging due to the different physicochemical properties that they possess. Both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have been used in single- and multi-class mycotoxin methods. ESI is more commonly used in multi-class multi-residue mycotoxins methods, although APCI has been reported to produce better results for the problematic trichothecene mycotoxins.[4,5] In ESI mode, the trichothecenes are prone to adduct formation, including sodium $[M+Na]^+$ and potassium $[M+K]^+$ adducts. These adducts produce a far greater signal response than the protonated molecular ions, which yield little to no signal response. However, using an ammonium buffer in the mobile phase overcomes this problem for the type-A trichothecenes (neosolaniol, diacetoxyscirpenol and T-2 toxin), and results in the formation of ammonium adducts $[M+NH_4]^+$ with a good MS response. In contrast, the type-B trichothecenes (nivalenol, deoxynivalenol, acetyldeoxynivalenol, and fusarenon X) do not form $[M+NH_4]^+$ adducts, although they are capable of forming acetate $[M+CH_3COO]^-$ and formate $[M+HCOO]^-$ adducts that produce an adequate signal response. In this study, ammonium formate (10 mM) was added to the aqueous mobile phase, resulting in the formation of $[M+HCOO]^-$ adducts for nivalenol and deoxynivalenol and $[M+H]^+$ ion for fusarenon X.

Acetonitrile and methanol were evaluated as the organic mobile phase eluent. Using acetonitrile resulted in the first eluting analyte, nivalenol, to exhibit poor peak shape (fronting) because of its high polarity and poor solubility in acetonitrile. Methanol, on the other hand, was found to give better peak shapes. Methanol was also found to improve the MS response, while the use of acetonitrile led to much lower signals. This is in agreement with previous reports.[6]

In the final method, separation of the mycotoxins, including α- and β-zearalanol, was achieved within 13 minutes on the Accucure aQ column (Figure 1). The use of rapid polarity switching allows all target analytes to be detected in a single run. Owing to their polarity, the type-B trichothecenes elute early in the chromatographic run, and are prone to matrix effects in the ion source.[3,7] To minimize ion source contamination and reduce the potential for matrix effects, the first 1.5 minutes of mobile phase eluent was diverted to waste.

QuEChERS Method Development

The QuEChERS approach is a commonly used sample preparation procedure for the analysis of mycotoxin residues. Acetonitrile is the preferred extraction solvent, as it extracts the widest range of mycotoxins and least amount of matrix components. Nivalenol, the most polar mycotoxin, is the only mycotoxin that does not give high recoveries using the QuEChERS approach.[8-11] This is due to the incomplete partitioning of nivalenol into the organic phase during the extraction/partitioning step of the QuEChERS procedure. Cleanup of the sample extracts is typically carried out by dispersive-SPE (dSPE) using primary secondary amine (PSA) and/or C18 sorbent. PSA is effective in removing organic acids, carbohydrates, and additional polar matrix components. C18 is effective in removing lipids and other lipophilic matrix components. If acidic compounds are included in the method, the sample pH needs to be sufficiently low to ensure the acidic compounds do not get retained on the PSA sorbent.

In this study, acetonitrile containing 2% formic acid and unbuffered QuEChERS salts were used to extract all the mycotoxins. The use of buffered extraction salts (acetate or citrate) was avoided, as this would raise the sample pH or require the use of higher amounts of acid to maintain a low pH of the extraction solvent. dSPE cleanup of the sample extracts was carried out with a combination of PSA and C18 sorbents. Using 2% formic acid in the extraction solvent was necessary to prevent the retention of ochratoxin A on the PSA sorbent. Using smaller amounts of acid resulted in lower recoveries for ochratoxin A.

Accuracy and Precision

Cereal, consisting of various grains, nuts, and seeds, was used as a representative sample matrix in this study. Cereal samples, determined to be free of any mycotoxin residues, were spiked at two concentrations (n=6 at each concentration) and extracted to obtain accuracy and precision data. The samples were fortified at 20 and 100 µg/kg and prepared according the experimental procedure described above. As outlined in Table 3, the majority of results were found be within an acceptable recovery range of 80–110 % with RSD values ≤ 10 %, demonstrating that the method is suitable for the analysis of mycotoxins in grain-based foods. Only nivalenol gave low recovery (≈70%), although the results were acceptable and reproducible (≤ 11.2%). It is well known that nivalenol cannot be completely extracted and partitioned into acetonitrile, and the extraction efficiency reported here is similar to that reported elsewhere.[8-11]

Table 3. Accuracy and precision data for the 16 mycotoxins fortified at two concentrations.

	20 µg/kg		100 µg/kg	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Nivalenol	71.4	11.2	67.2	6.5
Deoxynivalenol	106.7	4.1	97.0	2.8
3-Acetyldeoxynivalenol	100.4	3.9	97.2	1.9
Fusarenon X	96.3	3.9	96.2	3.8
Neosolaniol	100.5	3.3	99.4	2.0
Diacetoxyscirpenol	102.6	2.8	99.0	2.3
Alternariol	94.8	4.9	85.9	5.4
β-zearalanol	94.5	9.2	92.7	4.6
α-zearalanol	93.9	10.5	89.0	3.5
Zearalenone	92.4	9.4	87.6	4.5
Ochratoxin A	93.8	3.0	94.7	3.8
T-2 toxin	96.2	4.5	94.2	2.8
Aflatoxin B1	97.0	2.7	91.7	5.4
Aflatoxin B2	97.4	2.9	91.4	4.8
Aflatoxin G1	95.0	3.3	92.0	4.1
Aflatoxin G2	95.5	3.1	93.9	2.7

Linearity and Limit of Quantitation (LOQ)

Matrix-matched calibration curves of the 16 mycotoxins were prepared at concentrations of 5, 12.5, 25, 50, and 100 ng/mL and were found to give linear responses over the entire concentration range with correlation coefficients (R^2) typically greater than 0.995 (Figure 2 and Table 4).

The signal-to-noise ratio (S/N) at the lowest calibration level (5 ng/mL; 10 ng/g) was found to be >10 for all 16 compounds. Therefore, the LOQ was estimated to be ≤10 ng/g in this study.

Carryover

A methanol/water (50:50, v/v) solution containing no mycotoxins or IS was injected directly after the highest matrix-matched calibration standard to check for sample carryover. No analyte carryover was observed.

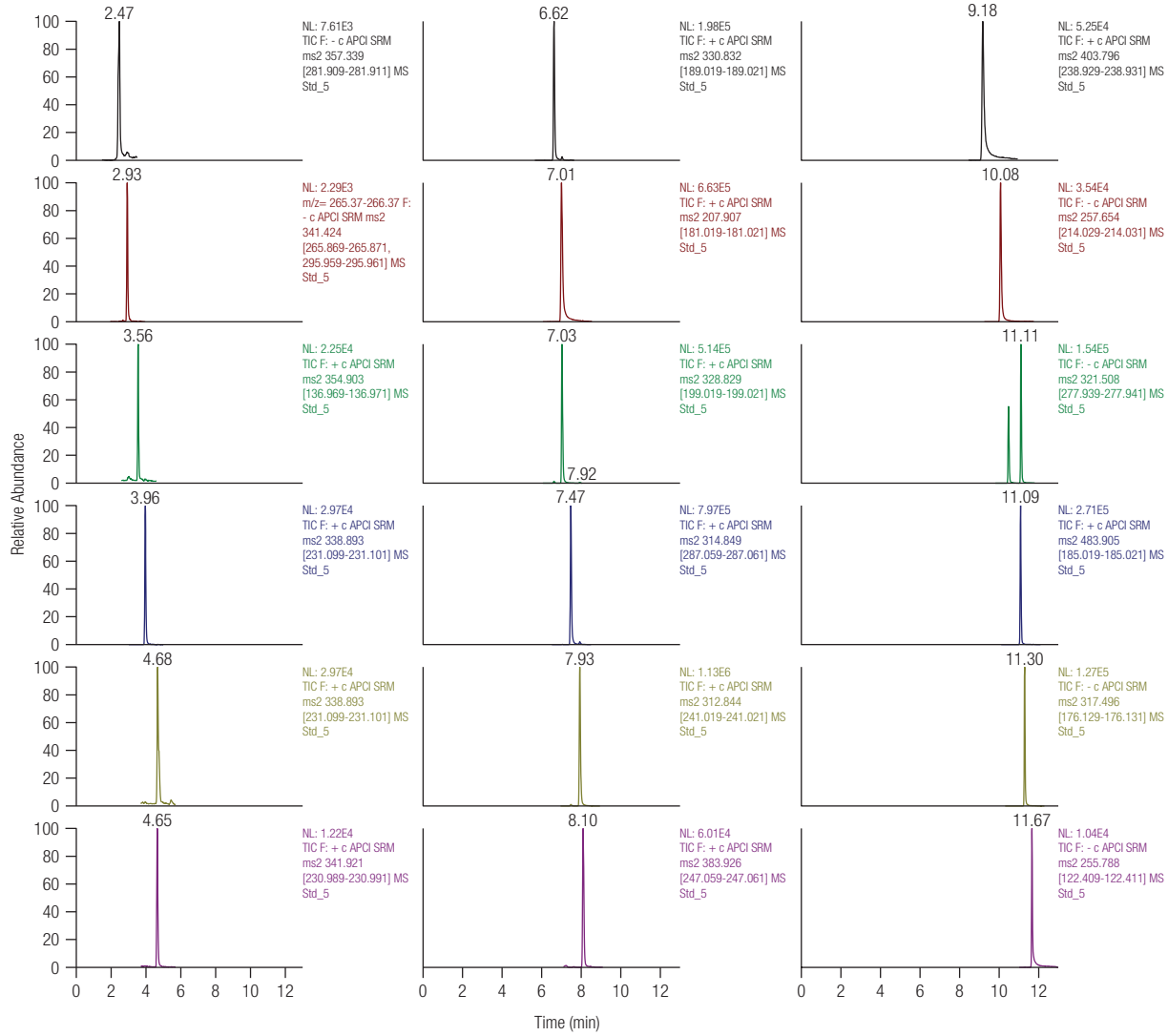

Figure 1. Chromatograms of the 16 mycotoxins and 3 internal standards at a concentration of 100 µg/kg.

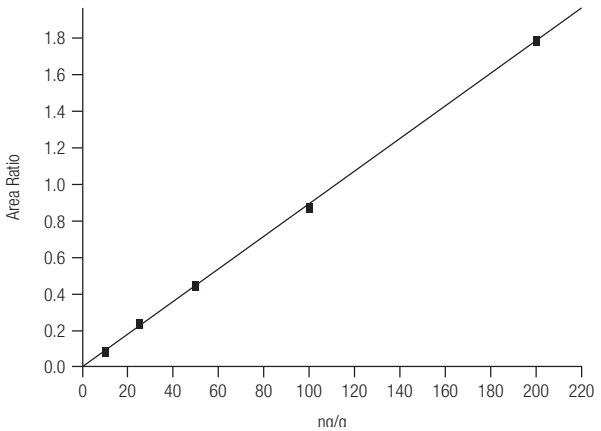

Figure 2. Example of a matrix-matched calibration curve (nivalenol).

Table 4. Linearity expressed as correlation coefficient (R^2).

Analyte	R^2
Nivalenol	0.9992
Deoxynivalenol	0.9963
3-Acetyldeoxynivalenol	0.9981
Fusarenon X	0.9989
Neosolaniol	0.9987
Diacetoxyscirpenol	0.9992
Alternariol	0.9993
β-zearalanol	0.9992
α-zearalanol	0.9993
Zearalenone	0.9991
Ochratoxin A	0.9996
T-2 toxin	0.9984
Aflatoxin B1	0.9985
Aflatoxin B2	0.9984
Aflatoxin G1	0.9981
Aflatoxin G2	0.9986

Conclusion

- A fast and easy QuEChERS method was developed for the analysis of 16 representative mycotoxins in grain-based food.
- Mycotoxin residues were extracted using acidified acetonitrile and unbuffered QuEChERS salts.
- Sample extracts were subsequently purified by dSPE using a combination of PSA/C18 sorbent.
- An LC-MS/MS method with a short run time was developed for the low level detection of mycotoxins.
- HPLC separation, including baseline resolution of α- and β-zearalanol, was achieved within 13 minutes on an Accucure aQ solid core column.
- Good linearity, accuracy and precision data, and satisfactory LOQs were obtained, indicating that the method is suitable for the analysis of mycotoxins in grain-based food.

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