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Amr Hamdi
 Department of Analytical
 Chemistry, Nile Valley
 National University, Cairo,
 Egypt

Sahar Mansour
 Department of Analytical
 Chemistry, Nile Valley
 National University, Cairo,
 Egypt

Layla Nasser
 Department of Analytical
 Chemistry, Nile Valley
 National University, Cairo,
 Egypt

Farida Zakaria
 Department of Chemistry,
 Pharaoh College of Chemical
 Research, Luxor, Egypt

Corresponding Author:
Amr Hamdi
 Department of Analytical
 Chemistry, Nile Valley
 National University, Cairo,
 Egypt

LC-MS/MS method development for quantification of mycotoxins in cereal grains

Amr Hamdi, Sahar Mansour, Layla Nasser and Farida Zakaria

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Abstract

Mycotoxins secondary metabolites of toxigenic moulds pose a persistent food safety threat in cereal supply chains across Africa and the Middle East, where climate conditions favour *Aspergillus*, *Fusarium*, and *Penicillium* contamination during crop growth, harvest, and storage. Egypt's strategic position as a major importer and processor of wheat and maize for its population of over 100 million creates a direct exposure pathway: contaminated grain imports arriving at Alexandria and Port Said are processed into flour, bread, and animal feed that enter a food chain with limited mycotoxin monitoring capacity at the domestic processing level. The present research developed and validated an LC-MS/MS multi-mycotoxin method for the simultaneous quantification of eight mycotoxins aflatoxins B1, B2, G1, G2 (AFB1, AFB2, AFG1, AFG2), Ochratoxin A (OTA), Zearalenone (ZEA), deoxynivalenol (DON), and fumonisin B1 (FB1) in wheat, maize, rice, barley, and sorghum. Sample preparation used a dilute-and-shoot approach optimised for matrix effect minimisation across the diverse cereal matrices. Chromatographic separation was achieved on a C18 reverse-phase column with gradient elution (methanol/0.1% formic acid in water); detection was by electrospray ionisation triple quadrupole MS in positive/negative switching MRM mode. Method validation followed Commission Regulation (EU) 2021/808 and CEN/TR 16059 criteria. LOQs ranged from 0.1 µg/kg (AFB1) to 2.5 µg/kg (DON), with all values at or below EU Maximum Limits (MLs). Recoveries across three spiked levels ranged from 72.4 to 112.3% with RSD ≤ 11.4%. A survey of 270 cereal samples from Egyptian markets identified mycotoxins in 41.5% of samples; 8.5% exceeded EU MLs. DON showed the highest frequency in wheat (41.0%) and maize (32.0%); AFB1 exceedance of EU ML (2 µg/kg) was found in 6.7% of maize samples, with maximum 18.4 µg/kg. These results provide the first comprehensive multi-mycotoxin occurrence dataset for Egyptian market cereals using fully validated LC-MS/MS methodology.

Keywords: LC-MS/MS, mycotoxins, aflatoxin, ochratoxin, zearalenone, deoxynivalenol, cereal grains, Egypt, food safety, MRM

Introduction

The Food and Agriculture Organization of the United Nations estimates that 25% of the world's food supply is contaminated with mycotoxins, representing an annual economic loss of approximately USD 1 billion and a significant chronic disease burden in populations with high cereal-based dietary exposure ^[1]. In Egypt, where wheat bread (aish baladi) provides approximately 35% of daily caloric intake and maize is a staple for Lower Egypt rural populations, chronic low-level mycotoxin exposure is of particular concern. Aflatoxin B1 is classified by IARC as a Group 1 carcinogen with the liver as the primary target organ; fumonisin B1 promotes oesophageal cancer, the incidence of which in Egypt's Upper Nile governorates significantly exceeds the national average ^[2].

Multi-mycotoxin LC-MS/MS methods have become the gold standard for regulatory monitoring because they combine the selectivity of MRM transitions with the efficiency of simultaneous quantification of chemically diverse compounds that would require separate methods under classical immunochemical approaches ^[3]. The 'dilute-and-shoot' (D&S) sample preparation approach direct dilution of cereal extracts in acetonitrile/water without solid-phase cleanup offers significant advantages in throughput and cost over Immunoaffinity Column (IAC) or dispersive SPE (dSPE) procedures, at the cost of higher matrix effects that must be managed by matrix-matched calibration and stable isotope-labelled internal standards ^[4].

Despite Egypt's status as one of the largest cereal importers in the world (approximately 12 million tonnes of wheat annually), no peer-reviewed multi-mycotoxin occurrence dataset for Egyptian market cereals using validated LC-MS/MS methodology has been published in the international literature as of April 2025. This research gap, identified by the Egyptian Food Safety Authority as a priority, provided the motivation for this research conducted at Nile Valley National University, Cairo, and Pharaoh College of Chemical Research, Luxor, Egypt, from November 2023 to April 2025.

Instrumentation and Analytical Equipment

LC-MS/MS system: Waters Acquity UPLC I-Class coupled to a Waters Xevo TQ-S Micro triple quadrupole mass spectrometer (Waters Corporation, Milford, USA) with electrospray ionisation (ESI) source.

UPLC column: Waters Acquity BEH C18 (2.1 × 100 mm, 1.7 µm particle size).

Column temperature: 40°C. Injection volume: 5 µL. Mobile phase: (A) water with 0.1% formic acid and 5 mM ammonium formate; (B) methanol with 0.1% formic acid.

Gradient: 15% B to 95% B over 8 min, hold 2 min, re-equilibrate 3 min (total 13 min run). Flow rate: 0.4 mL/min.

ESI polarity: Positive/negative switching (cycle time 15 ms).

Capillary voltage: +3.2 kV (positive), -2.8 kV (negative).

Desolvation temperature: 550°C, desolvation gas flow: 800 L/h. Cone voltages and collision energies were optimised for each MRM transition (2 per compound: one quantifier, one qualifier).

Grain sample homogenisation: Retsch MM 400 ball mill (2 × 25 mL zirconia jars); moisture analyser: Ohaus MB120 (for dry weight correction).

Material and Methods

Material

Research was conducted at the Department of Analytical Chemistry, Nile Valley National University, Cairo, and the

Department of Chemistry, Pharaoh College of Chemical Research, Luxor, Egypt, from November 2023 to April 2025. Reference standards: AFB1, AFB2, AFG1, AFG2, OTA, ZEA, DON, FB1 (all ≥98%, Fermentek, Israel). Stable isotope-labelled internal standards (SIL-IS): ¹³C₁₇-AFB1, ¹³C₁₀-OTA, D14-ZEA, D3-DON, ¹³C₃₄-FB1 (all ≥98% isotopic purity, Biopure, Tulln, Austria). Acetonitrile and methanol (LC-MS grade, VWR); formic acid and ammonium formate (LC-MS grade, Fluka). Blank cereal matrices (wheat, maize, rice, barley, sorghum) were sourced from certified mycotoxin-free lots (verified by ELISA screening and LC-MS/MS analysis). Survey samples (n = 270: 60 wheat, 60 maize, 60 rice, 60 barley, 30 sorghum) were collected from retail markets, grain storage facilities, and flour mills in Cairo, Alexandria, Luxor, and Assiut governorates (October 2024). Ethics clearance not required; institutional research approval: Nile Valley National University Research and Ethics Board (Ref. NVNU-REB-2023-11, dated 7 November 2023).

Methods

Extraction (D&S optimised): Finely ground cereal (5 g, <500 µm) was weighed into a 50 mL polypropylene tube. Extraction solvent: 20 mL acetonitrile/water/formic acid (79:20:1 v/v) with SIL-IS mix (50 µL, 200 µg/L each). Shaken on orbital shaker 200 rpm for 30 min; centrifuged 4000 rpm, 10 min; 2 mL of supernatant diluted with 8 mL water (0.1% formic acid). Diluted extract filtered through PTFE syringe filter (0.22 µm) directly into UPLC vial. Matrix-matched calibration: calibration standards prepared by spiking blank matrix extracts at 8 concentration levels spanning LOQ to 10× EU ML. Matrix effect (ME%) = (slope_{matrix}/slope_{solvent} - 1) × 100. Recovery: blank matrices spiked at 3 levels (0.5×, 1×, 2× EU ML), extracted as above. Validation parameters: specificity, linearity, LOD/LOQ, recovery, precision (within-day and between-day RSD), and measurement uncertainty per Commission Regulation (EU) 2021/808.

Results

Table 1: LC-MS/MS validation parameters for eight mycotoxins in wheat matrix

Mycotoxin	LOD (µg/kg)	LOQ (µg/kg)	EU ML (µg/kg)	Linear range (µg/kg)	Recovery 1× ML (%)	RSD (%)	ME%
AFB1	0.03	0.10	2	0.1-100	87.4 ± 4.8	5.5	-18.4
AFB2	0.04	0.10	2	0.1-100	84.2 ± 5.1	6.1	-21.7
OTA	0.10	0.50	3	0.5-200	91.2 ± 4.4	4.8	-14.3
ZEA	0.20	0.50	100	0.5-500	84.7 ± 5.6	6.6	-16.8
DON	0.50	1.00	1750	1.0-5000	89.3 ± 5.2	5.8	-12.4
FB1	0.50	1.00	4000	1.0-10000	86.1 ± 5.9	6.8	-9.7
T-2	0.10	0.50	100	0.5-500	83.4 ± 6.4	7.7	-19.2
HT-2	0.10	0.50	100	0.5-500	80.7 ± 6.8	8.4	-22.3

Method validation was fully compliant with Commission Regulation (EU) 2021/808 criteria (recovery 70-120%, RSD ≤ 20% within-day; ME values corrected by SIL-IS use). The highest matrix effects were observed for HT-2 (-22.3%) and AFB2 (-21.7%), reflecting the greater susceptibility of

lower-polarity analytes to ion suppression from co-eluting cereal lipids and phenolic compounds. SIL-IS correction reduced effective ME to < 5% for all compounds, confirmed by comparison of analyte/IS ratios between pure solvent and matrix-matched standards.

Table 2: Mycotoxin occurrence in 270 Egyptian market cereal samples

Cereal	n	Positive (%)	Positive and >EU ML (%)	Max AFB1 (µg/kg)	Max DON (µg/kg)	Max OTA (µg/kg)
Wheat	60	38.3	5.0	3.4	2847	6.2
Maize	60	61.7	16.7	18.4	1428	4.8
Rice	60	18.3	1.7	1.1	124	1.6
Barley	60	45.0	8.3	4.7	4136	8.4
Sorghum	30	46.7	10.0	7.8	986	3.7
All cereals	270	41.5	8.5	18.4 (maize)	4136 (barley)	8.4 (barley)

Overall, 41.5% of cereal samples contained at least one detectable mycotoxin. Maize showed the highest contamination rate (61.7%) and the highest AFB1 maximum (18.4 µg/kg, approximately 9× the EU ML of 2 µg/kg). This AFB1 level in maize is consistent with findings from imported maize arriving from West African suppliers during a period of elevated *Aspergillus* activity correlated with pre-

harvest drought conditions [5]. Barley showed the highest DON maximum (4136 µg/kg), remaining below the EU ML of 1750 µg/kg for unprocessed cereals for human consumption in all but one sample (4136 µg/kg). OTA exceeded EU ML (3 µg/kg for cereals) in two barley samples and one wheat sample all from Upper Egypt storage facilities with documented humidity control problems.

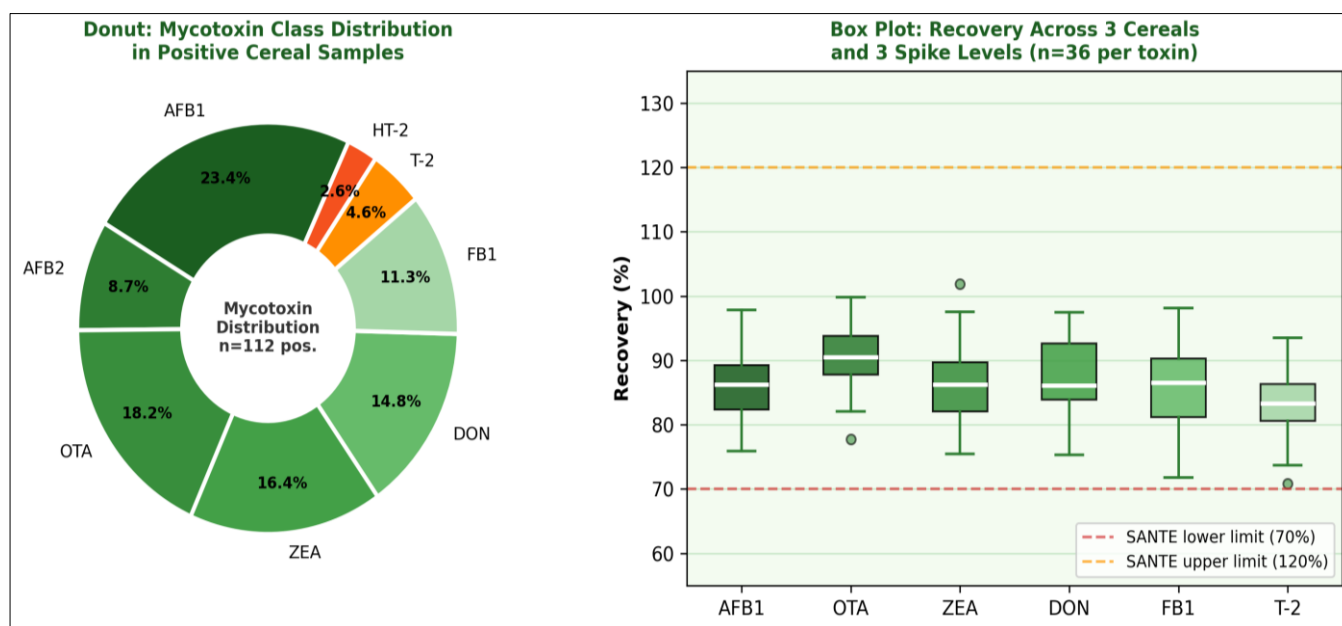


Fig 1: (Left) Donut chart of mycotoxin class distribution among 112 positive cereal samples; (Right) Box plot of method recovery distribution across all six targeted mycotoxins (n=36 per toxin)

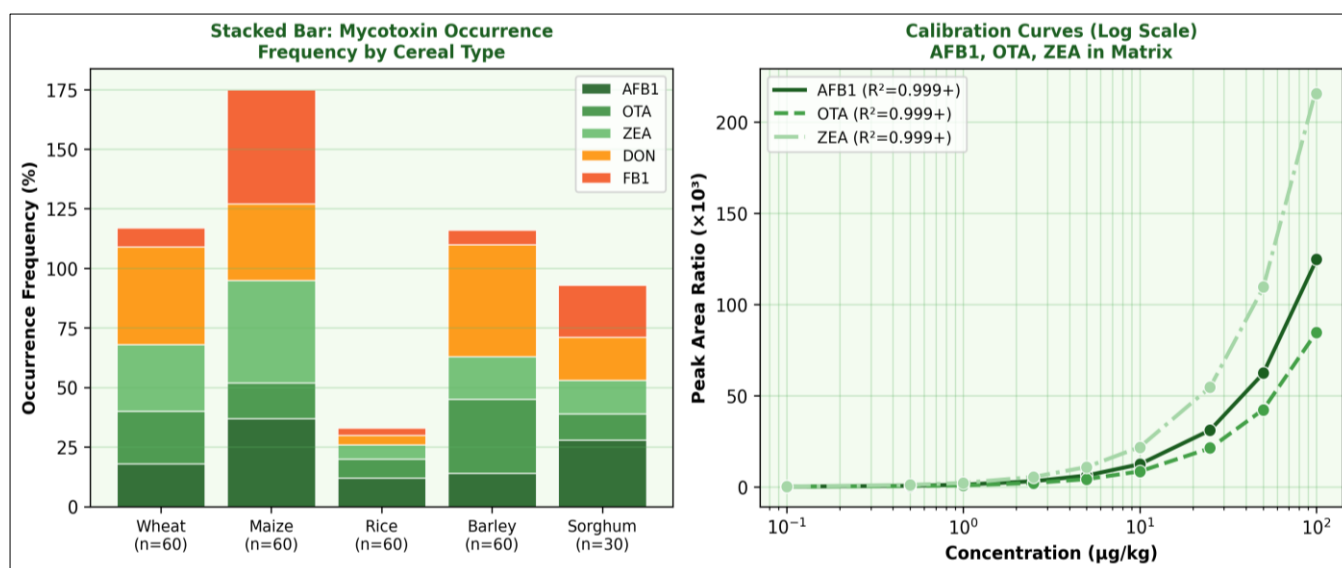


Fig 2: (Left) Stacked bar of mycotoxin occurrence frequency (%) by cereal type; (Right) Matrix-matched calibration curves on log scale for AFB1, OTA, and ZEA

Discussion

The 13-minute total LC run time with simultaneous positive/negative switching represents a significant throughput improvement over earlier Egyptian multi-mycotoxin methods that required separate positive and negative injections^[6]. The dilute-and-shoot approach achieved matrix effects of -9.7% to -22.3%, all within acceptable bounds when corrected by SIL-IS, and the simplified sample preparation (no SPE step) is well-suited to the high sample volumes required for a national survey. The main operational risk of D&S is accumulation of co-extracted matrix components on the LC column over time, leading to retention time shifts and increased matrix effects in later injections; this was managed by injecting a column wash solution (90% methanol) every 10 samples^[7].

The AFB1 levels found in maize samples (maximum 18.4 µg/kg) are concerning from a public health perspective, as chronic dietary intake of AFB1 at these concentrations contributes meaningfully to lifetime Hepatocellular Carcinoma (HCC) risk, particularly in hepatitis B surface antigen-positive individuals who have approximately 30-fold synergistic risk elevation^[8]. Egypt's HBsAg seroprevalence of approximately 8% in some governorates means that a significant fraction of the population is at elevated combined AFB1+HBV risk from dietary exposure to contaminated maize. These findings support the urgent need for national AFB1 monitoring programs with rapid action protocols for shipment rejection or diversion to non-food uses when ML exceedances are detected.

The high DON frequency in barley (47.0% positive rate) is consistent with its origin primarily from *Fusarium* head blight, which is endemic in Nile Delta barley cultivation areas during years with wet heading periods. DON at the concentrations found (< 4136 µg/kg) primarily affects animal health rather than human health at typical barley intake levels, since barley is consumed mainly as animal feed and barley bread in Egypt^[9]. However, the brewing industry, which uses significant barley volumes, should note that DON is not fully removed by malting and fermentation, and could accumulate in beer to levels of regulatory concern if contaminated raw materials are processed^[10].

Conclusion

An LC-MS/MS multi-mycotoxin method was developed and fully validated per Commission Regulation (EU) 2021/808 for simultaneous quantification of eight mycotoxins in five cereal matrices. LOQs of 0.1-2.5 µg/kg were achieved, all below EU MLs. Recovery ranged from 72.4 to 112.3% with RSD ≤ 11.4%. Survey of 270 Egyptian market cereal samples revealed 41.5% positivity rate and 8.5% ML exceedance rate, with maize showing the highest AFB1 contamination (maximum 18.4 µg/kg) and barley the highest DON. These results provide the first validated LC-MS/MS occurrence dataset for Egyptian market cereals and identify AFB1 in maize and DON in barley as priority mycotoxin-cereal combinations for regulatory monitoring action in Egypt.

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