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Morphed aflaxotin concentration produced by *Aspergillus flavus* strain VKMN22 on maize grains inoculated on agar culture

Viola O. Okechukwu [a], Abidemi P. Kappo [a], Patrick B. Njobeh [b], Messai A. Mamo [c,*]

[a] Department of Biochemistry, Auckland Park Kingsway Campus, University of Johannesburg, South Africa
[b] Department of Food and Biotechnology, PO Box 17011, Doornfontein Campus, University of Johannesburg, South Africa
[c] Department of Chemical Sciences, PO Box 2028, Doornfontein Campus, University of Johannesburg, South Africa

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ABSTRACT

This study identified and monitored the levels of aflatoxins (B_1 and B_2) produced by *Aspergillus flavus* isolate VKMN22 (OP355447) in maize samples sourced from a local shop in Johannesburg, South Africa. Maize samples underwent controlled incubation after initial rinsing, and isolates were identified through morphological and molecular methods. In another experiment, autoclaved maize grains were intentionally re-inoculated with the identified fungal isolate using spore suspension (106 spore/mL), after which 1 g of the contaminated maize sample was inoculated on PDA media and cultured for seven days. The aflatoxin concentrations in the *A. flavus* contaminated maize inoculated on culture media was monitored over seven weeks and then measured using liquid chromatography–mass spectroscopy (LC-MS). Results confirmed the successful isolation of *A. flavus* strain VKMN22 with accession number OP355447, which consistently produced higher levels of AFB_1 compared to AFB_2. AF concentrations increased from week one to five, then declined in week six and seven. AFB_1 levels ranged from 594.3 to 9295.33 μg/kg (week 1–5) and then reduced from 5719.67 to 2005 μg/kg in week six and seven), while AFB_2 levels ranged from 4.92 to 901.67 μg/kg (weeks 1–5) and then degraded to 184 μg/kg in week six then 55.33 μg/kg (weeks 6–7). Levene's tests confirmed significantly higher mean concentrations of AFB_1 compared to AFB_2 ($p \leq 0.005$). The study emphasizes the importance of consistent biomonitoring for a dynamic understanding of AF contamination, informing accurate prevention and control strategies in agricultural commodities thereby safeguarding food safety.

1. Introduction

Aspergillus flavus is a ubiquitous saprophytic filamentous pathogen that is abundant in various organic plant materials with high carbohydrate content (Abdel-Azeem et al., 2019). It thrives within optimal temperature ranging between 27 and 37 °C and requires a high relative humidity above 80 % for proliferation (Thathana et al., 2017). *A. flavus* synthesizes aflatoxins (AFs) including AFB_1 and AFB_2, which are potent mycotoxins known to contaminate crops (Okayo et al., 2020). The production of AFs by toxigenic *Aspergillus* species is influenced by various factors, including water activity (aw), temperature, substrate concentration, pH, and incubation time (Northolt and Bullerman, 1982; Omara et al., 2020). AF contamination in crops, particularly in legumes, cereals, nuts, and oil seeds, poses significant risks to human and animal health, as well as trade, due to the carcinogenic properties of AFB1 and its association with hepatocellular carcinoma (Okori et al., 2022).

Legumes and cereals, such as maize (Zea mays), are staple crops consumed worldwide (Temba et al., 2017). Maize is highly nutritious (Kaul et al., 2019) and serves as a vital source of income for millions (Mirzabaev et al., 2018; Norlia et al., 2018; Kaul et al., 2019). However, maize is susceptible to fungal deterioration and mycotoxin contamination, particularly by Aspergillus species (Temba et al., 2017; Achaglinkame et al., 2017). The proliferation of mycotoxigenic fungi in maize leads to a decline in nutritional content, discoloration, and reduced crop yields (Ur Rehman et al., 2021). Identification of *Aspergillus* section *Flavi*, which includes AF-producing species, like *A. flavus* and *A. paraciticus* relies on morphological and molecular approaches. However, conventional identification methods often lead to inaccuracies due to the close resemblance among these species, highlighting the need for accurate molecular techniques for differentiation (Norlia et al., 2018; Jenks et al., 2023).

The prevailing environmental conditions, characterized by elevated

* Corresponding author.
E-mail address: messaim@uj.ac.za (M.A. Mamo).

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temperatures, high humidity, erratic rainfall patterns, and frequent droughts could create conducive environments for the proliferation of mycotoxigenic fungi in food commodities worldwide, with a specific focus on Africa (Gbashi et al., 2019; Okechukwu et al., 2023). Consequently, routine monitoring of AF contamination in food commodities has become essential to ensure food safety, owing to the severe health implications associated with these mycotoxins (Gbashi et al., 2018). As a result, many nations have established regulatory thresholds for AF levels in food and feed products, necessitating routine monitoring for compliance (Udomkun et al., 2017; Soares et al., 2018; Meneely et al., 2023).

Analytical techniques such as liquid chromatography and mass spectrometry (LC-MS) play a crucial role in efficiently detecting and quantifying AFs in various matrices (Gbashi et al., 2019; Tebele et al., 2020; Okechukwu et al., 2023), while appropriate sampling protocols are essential for obtaining representative data. Timely monitoring facilitates regulatory adherence and enables proactive interventions in agricultural practices and storage conditions, thereby mitigating health and economic risks across the food supply chain (Gbashi et al., 2018). The main aim of this study was to evaluate the impact of time duration on AF production by *A. flavus* strain VKMN22 in maize grain inoculated on agar culture using the LC-MS analysis.

2. Experimental

2.1. Materials and reagents

In this study, the organic solvents and chloramphenicol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Other materials, such as disposable Norm-Ject 10 mL syringes with syringe Luer lock inlet filter units (25 mm, 0,22 μm PVDF), 2 mL chromatography amber vials, and glass beakers, were procured from Restek Corporation (USA). Polypropylene Falcon tubes (50 mL) were acquired from Fisher Scientific (Waltham, Massachusetts, USA). The AFB_1 and AFB_2 standards were acquired from the National Metrology Institute of South Africa (Pretoria, South Africa). Maize grains (1000 g) were purchased from a local shop in Johannesburg, South Africa and.

2.2. Fungal isolation procedure from maize seeds

The maize samples (700 g) were rinsed twice with distilled water to remove dust particles and moisten the seeds to promote growth. Subsequently, the maize samples were transferred into 1000 mL glass beakers, which were wrapped with parafilm and incubated in a controlled environment to stimulate fungal development. Upon the appearance of different colonies on the maize kernels after 20–25 days, the primary isolation criteria included visible mycelial development and spores exhibiting greenish-like coloration. Subsequently, six colonies suspected to be aflatoxigenic *Aspergillus* species were aseptically isolated and inoculated on PDA media fortified with 100 mg/L chloramphenicol for the inhibition of bacterial growth. The Petri dishes were securely sealed with parafilm and incubated at 27 °C for 10 days.

2.3. Identification of a. flavus isolate

The *A. flavus* strain VKMN22, utilized in this study was isolated from the maize grain samples purchased from a local shop in Johannesburg, South Africa. The fungal species were identified through macroscopic and microscopic methods using the identification method described by Adelusi et al. (2022) and subsequently confirmed via molecular analysis. Genomic Deoxyribonucleic acid (DNA) was extracted from each isolate using the Bacterial/Fungal DNA Extraction Kit (Zymo Research, D6005, Irvine, California, USA) following the manufacturer's instructions. Approximately 160 mg of fungal mycelia was suspended in isotonic water and lysed using ZR BashingBead™ lysis tubes containing BashingBead buffer solution. The extracted DNA was quantified using an ND-1000 spectrophotometer (NanoDrop Technologies) and adjusted to a concentration of approximately 50 ng/μL. The DNA bands were confirmed via electrophoresis on a 2 % agarose gel, and the samples were stored at −20 °C for further analysis.

Each isolated DNA fragment was selectively amplified within the Internal Transcribed Spacer (ITS) gene region using the primer combination ITS-1: 5-TCC GTA GGT GAA CCT GCG G-3′ (forward) and ITS-4; 5-TCC TCC GCT TAT GC-3′ (reverse) as previously reported by White et al. (1990). Each PCR reaction contained of 12.5 μL of Red Taq Ready Mix obtained from Sigma-Aldrich (Germany), 0.8 μL of the extracted DNA sample, 0.3 uL of each primer used (i.e., ITS1 and ITS4), and 0.5 μL of dimethyl sulfoxide (DMSO), and 9.6 μL of double-distilled water (ddH_2O) was prepared, resulting in a total volume of 24 μL. A negative control was also prepared, including all reagents, except DNA.

PCR amplification was performed using a ProFlex 32-well PCR System (ThermoFisher Scientific, Singapore), The initial denaturation of DNA was performed for 2 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 50 °C for 30 sec, and primer extension at 72 °C for 1 min. Following the final extension step at 72 °C for 10 mins holding at 4 °C. Afterward, PCR products were purified using a DNA ZR-96 sequencing clean-up kit (Applied Biosystems, Foster City, CA, USA) to eliminate residual primers. The purified PCR products were subsequently sequenced in both forward and reverse gene regions using an ABI 3130 × 1 Genetic Analyzer (Thermo Fisher Scientific, Massachusetts, USA). The consensus DNA sequences were investigated using BLAST https://www.ncbi.nlm.nih.gov/Blast.cgi to identify the species name.

The 18S rRNA gene sequences obtained from the GenBank database were used as the basis for performing data alignments and phylogenetic analysis using the ClustalW algorithm on the EMBL-EBI website (accessible at https://www.ebi.ac.uk/Tools/msa/clustalo). The phylogenetic tree was constructed using the MEGA 11 software, following the methodology described by Kumar et al. (2016) and Tamura et al. (2021), by using the maximum likelihood approach to measure the evolutionary distance and clustering. Bootstrap values based on 1000 replications were chosen as the parameter for phylogenetic tree construction, with branches having less than 50 % site coverage being collapsed (Felsenstein, 1985; Russo and Selvatti, 2018). The constructed phylogenetic tree was used to assess the evolutionary relationship between the *A. flavus* strain VKMN22 isolated in this study and its relatives in the GenBank database. The DNA sequence was subsequently submitted to GenBank and assigned the accession number (OP355447). The outgroup pecies were *Fusarium equiseti* strain RM271 and *Fusarium equiseti* strain SPJ22.

2.4. Extraction procedures for liquid chromatography-mass spectrometry (LC-MS) analysis

Maize samples (approximately 25 g) were pretreated in 1 % sodium hypochlorite, rinsed as described by Li et al. (2021), milled, and then sterilized using an autoclave for 15 min at 121 °C. Subsequently, the milled maize sample was intentionally contaminated with a fungal isolate aliquot using (10^6 spore/mL) spore suspension. Then, 1 g of the contaminated maize sample was inoculated on solidified PDA media (triplicate) and further incubated at 27 °C for AF formation. A total of 21 plates were prepared.

The fungal isolate was extracted using a solvent extraction method with slight modifications following the procedure described by Dada et al. (2020). At weekly intervals, three plate cultures were removed from the incubator, plugged (including the media), and deposited in 100 mL glass beakers. The extraction solution (20 mL), comprised of acetonitrile/water/acetic acid in a ratio of 79:20:1 (v/v/v), was added to the beakers and vigorously shaken for 30 min at room temperature using a Labcon shaker (Labcon, USA). The solutions were separated using Whatman No. 1 filter paper, followed by an additional filtration step through a 0.22 μm syringe nylon filter. Subsequently, the resulting

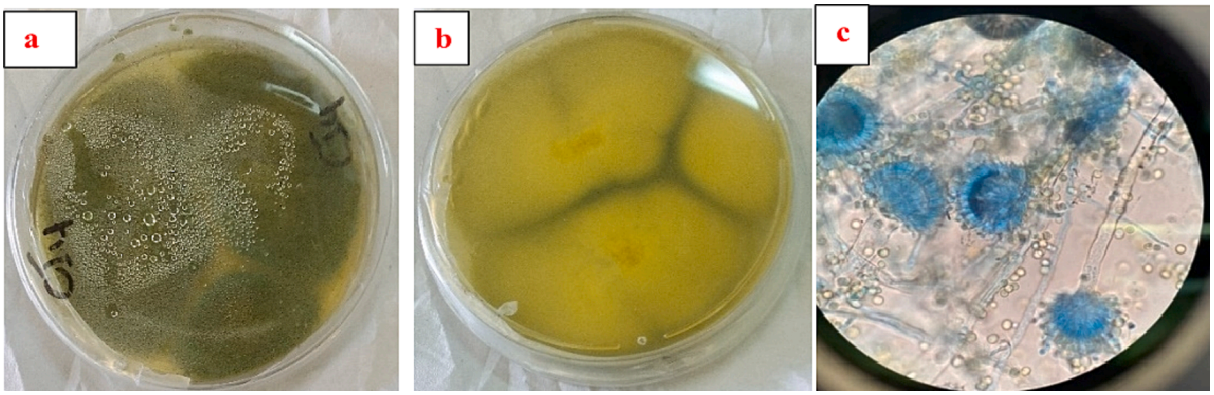

Fig. 1. Morphological characteristics of *A. flavus* VKMN22. Macroscopic features of the isolate grown on PDA, showing (a) mycelial growth on the front side, (b) the reverse side of the isolate and (c) microscopic characteristics of the isolate under a 100X lens of basic biological light microscopic.

filtered solutions were transferred into LC-MS vial bottles (1.5 mL) for analysis.

2.5. Validation approach

The performance of the technique was validated for two selected AFs (B_1 and B_2) produced by *A. flavus* in maize plated on solidified PDA media, following the acceptability parameters set by the European Commission (2006a). The validation parameters evaluated were matrix effects, linear range, limit of detection (LOD), limit of quantification (LOQ), recovery, and selectivity.

Matrix-matched standard curves were generated using the solution extracted from sterile milled maize plated on PDA media (i.e., blank solution), given that the samples were cultured with maize and plated on PDA media. Neat standard and matrix-matched calibration curves were generated to assess the influence of the matrix effects on the analyzed samples. The 12-point linear calibration curves in the 0.9–2000 μg/kg range were generated. The calibration curves comprised both neat standards and matrix-matched calibration curves comprising two AFs (i.e., AFB_1 and AFB_2). Subsequently, the neat standard was used to generate standard concentrations for external and instrument calibration, whereas matrix-matched standard curves were used to quantify the levels of AFs in the samples.

Matrix effects (ME) for AFs were calculated using Arroyo-Manzanares et al. (2018), signal enhancement, or suppression techniques. The matrix effect was determined by evaluating the slopes of the calibration curves of both the matrix-matched and neat standards, as shown in Eq. (1).

$$\mathrm{ME} = \frac{\text{slope of m} - \text{slope of n}}{\text{Slope of n}} \mathrm{X}100 \tag{1}$$

ME indicates the matrix effect, and the slopes of m and n represent the standard matrix-matched calibration curve and neat standard curve slope, respectively.

The LOD and LOQ were calculated based on the signal-to-noise ratio (S/N) multiplied by 3 and 10, respectively, as indicated by Eqs. (2) and (3):

$$\mathrm{LOD} = 3.3\,\mathrm{X}\frac{\text{Concentration}}{\text{Signal} - \text{to} - \text{Noise}} \tag{2}$$

$$\mathrm{LOQ} = 10\,\mathrm{X}\frac{\text{Concentration}}{\text{Signal} - \text{to} - \text{Noise}} \tag{3}$$

A recovery test was conducted on the combined extract of blank milled maize and PDA media (blank), and recovery was determined by spiking the blank sample with 50 μg/kg (low) and 100 μg/kg (high) of AFs followed by subsequent injection for analysis. The recovery value as a percentage was determined by dividing the measured concentration by the theoretical spike concentration and multiplying the value by 100, as given in Eq. (4) (Arroyo-Manzanares et al., 2018; Tebele et al., 2020).

$$\text{Recovery} = \frac{\text{measured concentration}}{\text{theoretical spike concentration}} \mathrm{X}\,100 \tag{4}$$

The selectivity of the method was evaluated by analyzing the standards and contaminated samples, followed by monitoring the transition of each analyte at a suitable retention time.

2.6. Instrumentation for liquid chromatography-mass spectrometry

A Shimadzu LC-MS 8040 instrument (Shimadzu Corporation, Tokyo, Japan) was used to analyze the AFs. Chromatographic separation was performed using a Phenomenex KINETEX® C18 LC column (2.6 μm, 2.1 mm x 100 mm) (Core-Shell Technology) while maintaining a constant oven temperature of 40 °C. The LC-40B XR solvent delivery module chromatograph was connected to an SIL–40C Nexera autosampler, DGU–403 degassing unit, and a CTO–40S column oven. The mobile phase consisted of an aqueous phase containing 0.1 % formic acid in deionized water (solvent A) and an organic phase composed of a mixture of acetonitrile and methanol (50:50, v/v) with 0.1 % formic acid (solvent B), delivered at a constant flow rate of 0.2 mL/min, and a sample injection volume of 10 μL. The elution gradient program commenced with 0.5 min at 20 % solvent B and increased linearly to 95 % solvent B for 8.5 min, which was then maintained for 2 min. The total elution gradient run time was 15 min. The column was reconditioned with 10 % solvent B for 1 min to prepare for each subsequent run, followed by a 5-minute normalization period. Electron spray ionization (ESI) was used as the ionization source and operated in the positive mode at an event time of 0.206 sec. Data were acquired using the multiple reaction monitoring (MRM) technique under optimized MS conditions. The interface conditions were set at a desolvation line (DL) temp. At 250 °C, the nebulizing gas flow rate was 3.00 L/min, the heat block temperature was 400 °C, and the drying gas flow rate was 15 L/min.

2.7. Statistical analysis

The concentration of each analyte was determined by averaging the values obtained from triplicate measurements for each sample over a period of seven weeks. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Origin 9.0 software (OriginLab Corporation, Northampton, Massachusetts, USA). Levene's test evaluated the statistical significance ($p \leq 0.05$) differences. Descriptive statistics such as the standard deviation (Std Dev), mean and standard error (Std Error) were calculated to assess the dispersion and central tendencies of the sampling information and AF concentrations. among different weeks.

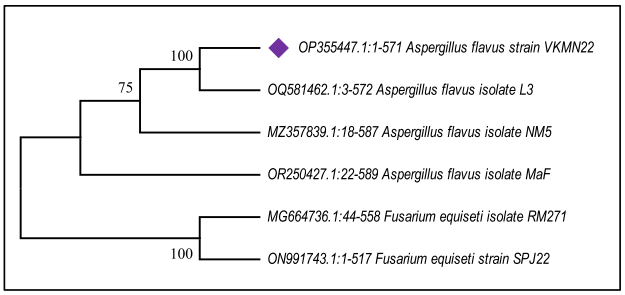

Fig. 2. Molecular phylogeny of *A. flavus* strain VKMN22 isolated from maize seeds based on the ITS spacer of ribosomal RNA region. Bootstrap values, expressed as percentages, are indicated at internal nodes.

3. Results and discussions

3.1. Identification of a. flavus strain VKMN22

For the identification analysis, both morphological and molecular approaches were used for accurate identification due to fungi complex and ubiquitous nature as well as unstable characteristics (Basson et al., 2019).

3.1.1. Morphological characterization of the isolate

Only the greenish-like spores were collected and transferred to Petri dishes containing freshly prepared PDA media for macroscopic examination of the pure culture. The microbiota proliferated on the PDA media, and after three days, sporulation initiated in the middle of the colony and gradually spread outward, eventually covering the entire surface (Fig. 1a). The isolates produced conidia with a yellowish-to-olive colour, which ultimately dominated colony morphology. A distinct white border formed around the sporulating mycelia as the sporulation extended outward. By days 7–10, as the mycelia kept sporulating and generating additional conidia, they completely covered the white border. Additionally, all isolates displayed clear transparent exudates in water droplets and a cream colour on the backside (Fig. 1b). The *A. flavus* isolates VKMN22 exhibited greenish colony morphology that expanded radially from the spot of inoculation. As the colonies grew, they gradually increased in size due to the development of mycelia, and the centre became rough and floccose in appearance.

For microscopic examination, distinctive features were observed in the *A. flavus* isolate VKMN22. The colonies exhibited a biseriate growth pattern, wherein phialides extended radially from the metulae. These metulae were on sub-globose or globose vesicles of different sizes (Fig. 1c). Notably, the metulae entirely covered the surface of the vesicles. The isolate conidia exhibited a globose shape characterized by thin walls and a smooth texture. They were observed to be non-pigmented and possessed a rough exterior. The conidiophores, on the other hand, were thick, unbranched structures and non-pigmented.

The initial step for fungal isolation involved culturing the fungi colonies from maize seeds on culture media to facilitate colony growth and observe their morphological characteristics. These taxonomic descriptive features were used as the initial criteria for fungal identification. Similar features have been reported in previous studies by Ekwomadu et al. (2018), Adelusi et al. (2022), Khan et al. (2020), Thathana et al. (2017) and Compaoré et al. (2021). However, it was acknowledged that the isolation and morphological identification were insufficient to fully establish the fungal isolate's identity at species level. A molecular technique is extensively used for accurate identification and detailed characterization of the genus *Aspergillus* (Norlia et al., 2018), with the ITS rDNA region sequence serving as an essential tool for discerning fungal species from environmental sources (Alsohaili and Bani-Hasan, 2018; Talukdar et al., 2021).

3.1.2. Molecular characterization and identification of a. flavus strain VKMN22

The fungal isolate *A. flavus* strain VKMN22 (OP355447) was successfully identified via molecular analysis of the internal transcribed spacer (ITS) gene sequence. In order to establish the correlation between *Aspergillus* spp and *A. flavus* strain VKMN22, a phylogenetic tree was constructed based on the ITS gene sequence of VKMN22 (OP355447) in comparison to other species of *Aspergillus* (Fig. 2). Notably, *A. flavus* strain VKMN22 exhibited a distinct clustering pattern with A. *flavus strains* L3, with 100 % identity and 75 % identity with *A. flavus* isolates NM5 and MaF. The phylogram was rooted using *F. equiseti* strain RM 271 and *F. equiseti* strain SPJ22 as the outgroup. The phylogenetic evaluation of the fungal isolates in this study using rRNA genes showed that recombination and mutation are the main causes of genetic diversity in many fungi strains found in agricultural crops (Taylor et al., 2017).

Additionally, the use of ITS rRNA sequence genes in performing the phylogenetic analysis is justified as they are adequately conserved, widely dispersed, functionally constant, and of sufficient length to provide a comprehensive understanding of evolutionary relationships, as noted by Alsohaili and Bani-Hasan (2018).

3.2. Validation approach

Two solvent phases were used: an organic phase (methanol and acetonitrile in a 50:50 v/v ratio) and a mobile phase (ddH_2O) in liquid chromatography coupled with mass spectrometry analysis. These solvents were selected as the organic phase because they influenced the chromatographic separation quality and facilitated analyte ionization (Cortese et al., 2020). The mobile phase was used to achieve optimal conditions and regulate the ionization extent of AFs in ESI + mode since AFs are reported to ionize better in positive mode (Miró-Abella et al., 2017; Leite et al., 2023). Moreover, including buffers in the mobile phase and the organic phase enhances ionization efficiency and improves the peak shapes (Luo et al., 2023).

The acquired results from the validation of the methods used in the LC-MS technique and sample extraction for determining AF levels in contaminated milled maize samples inoculated on PDA media are shown in Table 1. The evaluation of matrix effects (ME) was determined using the standard calibration curves; the ionisation enhancement for AFB_1 and AFB_2 were found to be 327 % and 236 %, respectively. Panuwet et al. (2016) reported that matrix-matched effect values exceeding 100 % indicate ion enhancement, while values below 100 % indicate signal suppression. Calibration curves were generated for AFB_1 and AFB_2 at different concentration levels ranging from 0.9 to 2000 μg/kg to evaluate linearity. The results of the validation demonstrated a satisfactory coefficient of determination ($R^2 > 0.999$). Standard calibration curves (refer to Supplementary Figure 1) were constructed by plotting the peak areas of the optimum emission lines against the corresponding concentrations using least squares linear regression.

The validation of the method was confirmed by comparing the LODs and LOQs obtained with the maximum allowable limits set by the European Union (EU) for AFs in maize, which are 2 μg/kg for AFB_1 and 4 μg/kg for total AFs. The LOD and LOQ values obtained for AFB_1 are 0.2

Table 1
Optimized analytical parameters for the LC-MS method.

AFs	Matrix effect (%)	Linear range (μg/kg)	R^2	Slope	LOD (μg/kg)	LOQ (μg/kg)	Recovery % 50	100	Retention Time (mins)	
B_1	327	0.98–2000	0.9999	7450.48	0.2	0.5	80	87.9	6.989	1.1
AB_2	236	0.98–2000	0.9999	6405.88	0.4	1.1	69	75	6.723	1.1

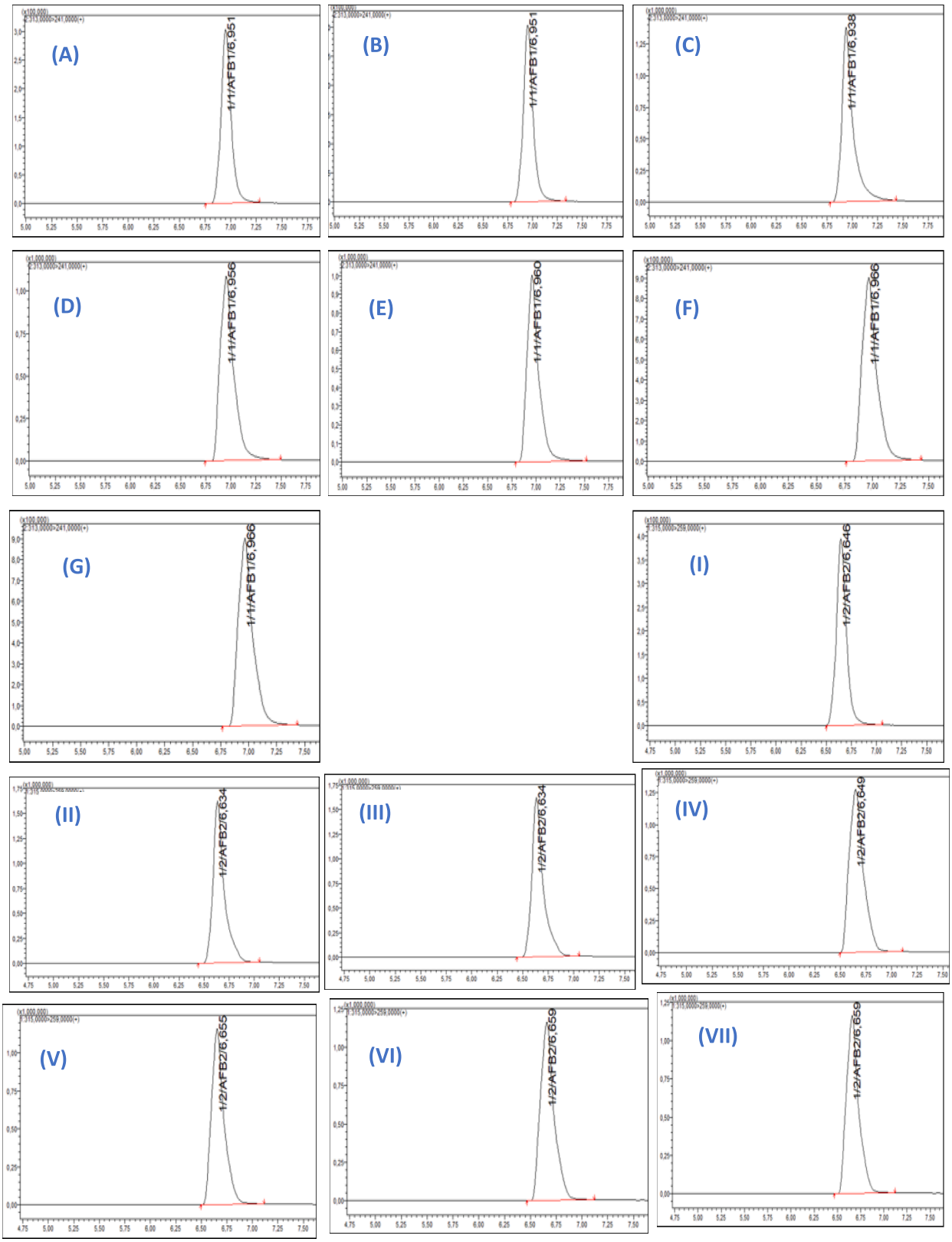

Fig. 3. The LC-MS chromatogram of multiple reaction monitoring (MRM) for AFB_1 (A to G) and AFB_2 (I to VII) standards with quantifier ions.

and 0.5 μg/kg, while the values for AFB_2 are 0.4 and 1.1 μg/kg, respectively (Kara et al., 2015). These LOD and LOQ estimations corresponded with established values reported by Tebele et al. (2020). The recovery values at spiking levels 50 and 100 (μg/kg) for AFB_1 (80 and 87.9 %) and AFB_2 (69 and 75 %), respectively, fell within the established limits (70–120 %) set by the European Commission (2006a). Overall, the methods validation process ensures the reliability and validity of the analytical results for assessing AFB_1 and AFB_2 levels in maize grains contaminated by *A. flavus*.

The method's selectivity was evaluated by examining the specificity

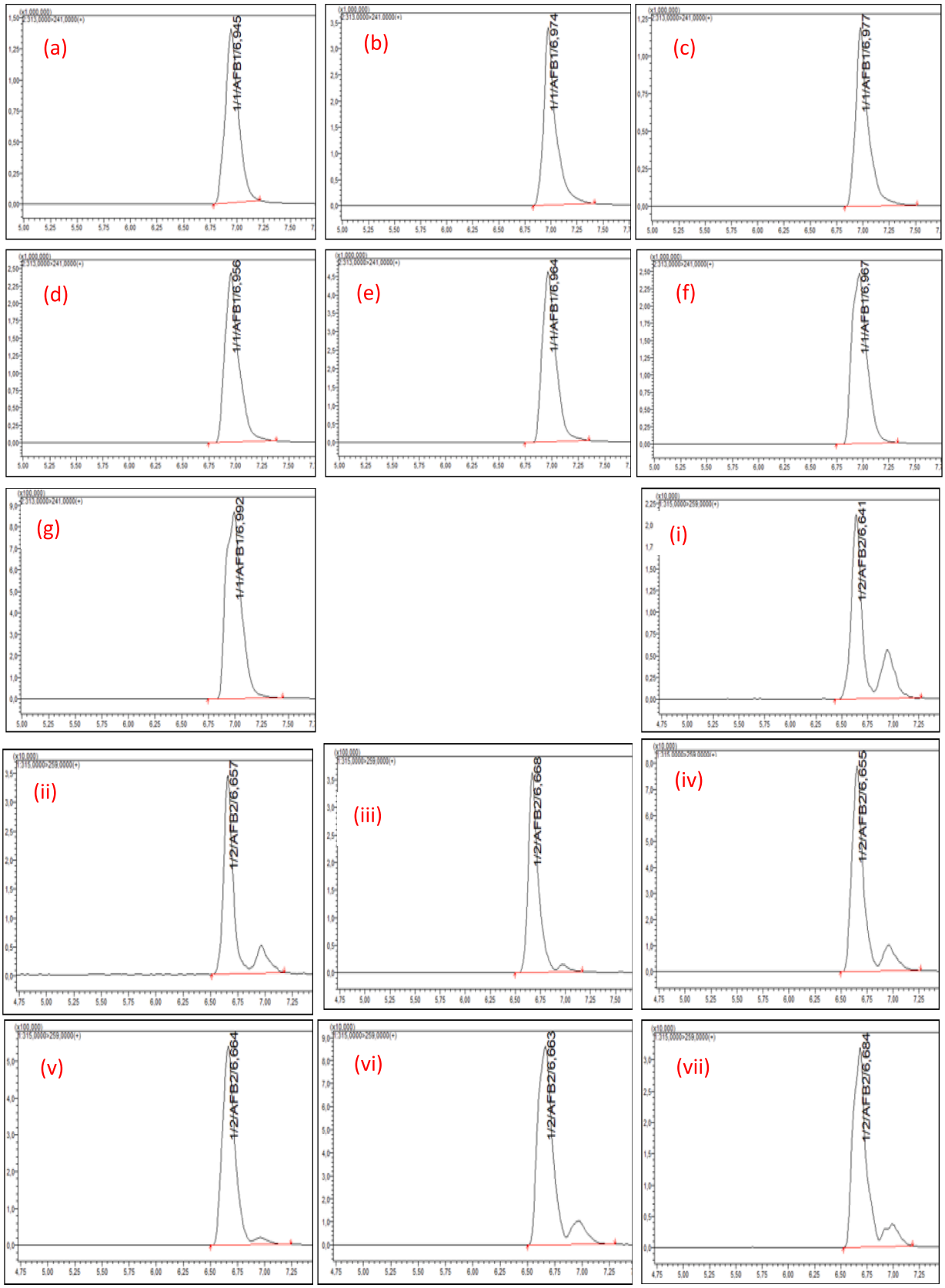

Fig. 4. LC-MS chromatograms with ion quantifiers obtained from *A. flavus* VKMN22 contaminated maize grains on culture media of AFB_1 (a to g) and AFB_2 (**1 to vii**) over the period of seven weeks.

Table 2
Mean concentrations of AFB_1 and AFB_2 in spiked maize samples obtained over 7 weeks (no of plates per week = 3).

Time (weeks)	Mean (µg/kg)	Std Dev	Std error	95 % confidence interval for Mean	
				Lower Bound	Upper bound
AFB_1					
Week 1	594.33	4,04	2,33	584.29	604.37
Week 2	844.00	25.51	14.73	584.29	907.38
Week 3	2326.67	4.16	2.40	2316.32	2337.01
Week 4	4982.67	2.08	1.20	4977.50	4987.84
Week 5	9295.33	62,23	35.92	9140.75	9449.92
Week 6	5719.67	62.93	36.2	5563.34	5876.00
Week 7	2005.00	3.46	2	1996.39	2013.61
$AFFB_2$					
Week 1	4.92	0.00	0.00	5	5
Week 2	22.33	0.58	0.33	20.90	23.00
Week 3	48.6437	1.53	0.88	44.87	50.00
Week 4	133.67	1.15	0.67	130.80	135.00
Week 5	901.67	3.21	1.86	893.68	904.00
Week 6	184.00	2.65	1.53	177.43	186.00
Week 7	55.33	4.04	2.33	45.29	60.00

Table 3
Levene's test showing the significance level of AFs produced by *A. flavus* over 7 weeks.

AFs	Statistics	Mean squares	df1	df2	F-value	p-value
AFB_1	8306.437	1384.437	6	14	8.832	0.00041
AFB_2	22.772	3.795	6	14	5.476	0.004

of retention times and detecting each target analyte's Multiple Reaction Monitoring (MRM) transition. The retention times of AFB_1 and AFB_2 were positioned at 6,989 and 6,723, respectively. The LC-MS chromatogram of multiple reaction monitoring (MRM) of AFB_1 and AFB_2 standards with quantifier ions are shown in Fig. 3, while the curves for the contaminated milled maize samples incubated on PDA media at 27 °C are displayed in Fig. 4. The obtained results showed that no co-eluting or interfering peaks were detected at the appropriate retention times, thereby allowing for the selective determination of AFB_1 and AFB_2 produced by *A. flavus* strain VKMN22.

3.3. The statistic analysis of AFB_1 and AFB_2 produced by a. flavus on maize grains inoculated on PDA media over a period of seven weeks

The results in Table 2 provides the mean concentrations of AFB_1 and AFB_2, along with corresponding standard deviation and errors, including the 95 % confidence intervals, measured weekly over a duration of seven weeks. Our results showed that the spiked maize samples deposited on PDA tested positive for the analysed AFs. The homogeneity of variance using Levene's test in Table 3 confirms the significance level ($p \leq 0.05$) of AFs (B_1 and B_2) mean concentrations obtained over 7 weeks. The results showed that the concentrations of AFB_1 and AFB_2 produced by *A. flavus* strain VKMN22 were statistically different ($p < 0.05$), as depicted in the supplementary Table 4.

3.4. The occurrence of AFB_1 and AFB_2 produced by a. flavus on maize grains inoculated on PDA media over a period of seven weeks

The mean concentration of AF produced by *A. flavus* strain VKMN 22 over the period of seven weeks is depicted in Fig. 6a and b, also in the supplementary Table 4. The results showed that formation AFs (B_1 and B_2) started at week one. AFB_1 and AFB_2 concentrations exhibited a discernible linear increase before reaching a plateau, followed by a subsequent decline. The concentrations of AFB_1 produced by *A. flavus* increased proportionally from weeks 1 to 5, ranging from ranging from 594.33 to 9295.33 µg/kg and decreased linearly from 5719.67 to 2005 for week 6 and 7, respectively (see Fig. 6a). The concentration of AFB_2 increased from 4.92 to 901.67 µg/kg and subsequently reduced to 184 and 55.33 µg/kg. A similar pattern was observed for the mean concentrations of AFB_2 over the seven weeks; however, the amount of AFB1 produced tends to be higher than that of AFB_2. Giray et al. (2007) also detected higher levels of AFB_1 than AFB_2 in wheat samples in some Turkey regions.

In this study, higher levels of AFB_1 formation were detected over 7 weeks compared to AFB_2, confirming the report of (Xie et al., 2018), that the branch biosynthesis pathway responsible for AFB_1 production is more active and dominant than that of AFB_2 branch biosynthesis pathway. The optimal temperature range for maximum AF formation is reported to be between 25 and 35 °C (Adelusi et al., 2023, Hassane et al., 2017). The same pattern was observed in AFB_1. The simultaneous formation of AFs by *A. flavus* highlighted the influence of ecological factors such as substrate type and amount, temperature, pH, water activity, and culture age on AF formation from toxigenic *A. flavus* (Phan et al., 2021). In week 5, the analysis of samples revealed the highest mean concentrations of AFB_1 and AFB_2. This observation indicates that during this

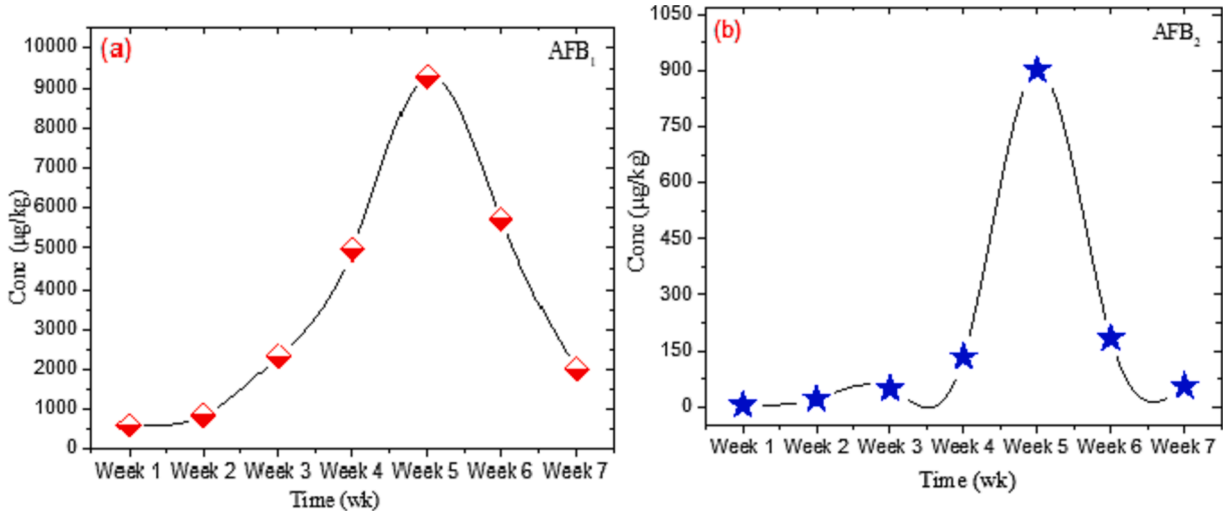

Fig. 6. Graphs of mean concentrations of (a) AFB_1 and (b) AFB_2 quantified over the period of 7 weeks.

specific timeframe, the conditions were conducive to the synthesis and accumulation of these AFs (Mamo et al., 2017; Schamann et al., 2022).

Subsequently, a linear reduction in mean concentrations of AFs, specifically AFB_1 and AFB_2, was observed in weeks 6 and 7, respectively. The levels of AFB1 decreased from 9295.33 μg/kg in week 5 to 5719.67 μg/kg in week 6, and further reduced to 2005 μg/kg in week 7. Similarly, the concentrations of AFB_2 decreased from 901.67 μg/kg to 184 μg/kg in week 6 and then to 55.33 μg/kg in week 7, following the pattern observed in week 5. As reported by Xie et al. (2018), either the majority of AFs were likely detoxified by the activity of cell-free extracts and mycelia of *A. flavus*, or were degraded during cell metabolism by cellular enzymes such as peroxidase (Alberts et al., 2009, Xing et al., 2017) and laccase (Adebo et al., 2017) into less toxic or non-toxic compounds. These enzymes can specifically target the chemical structure of AFs, leading to their degradation. Studies by (Doyle and Marth (1978a), Pitt (1993) as well as Hamid and Smith (1987) have demonstrated that AF-producing moulds have the capability to biodegrade their own synthesized AF. In addition, according to Dix et al. (2012), the onset of mycelial lysis, a significant increase in residue nitrogen, a rapid increase in pH, temperature, availability of other nutrients and sucrose depletion all occurred concurrently with AF degradation, as observed in weeks 6 and 7. Additionally, specific environmental and genetic conditions intrinsic to the mould could potentially enhance the activity of enzymes responsible for the degradation process of AF level (Doyle and Marth, 1978b).

Fungal contamination in crops, including maize, can occur during various stages, such as farming, transportation, and storage. The prevalence of fungal contamination can vary across farms within the same region (Koskei et al., 2020), particularly under adverse growing conditions. In South Africa, local farmers predominantly cultivate crops in rural areas, and traders import few crops, such as cereals and nuts, from neighbouring countries, including Malawi, Mozambique, Kenya, and Zambia. These crops may be consumed raw or partially cooked, and they are also used as animal feed without thorough cleaning or complete cooking procedures. The contamination of maize crops by AF poses a significant risk to the health of humans and animals (Rizzo et al., 2021). Therefore, monitoring AF contamination at an early stage is of great importance in safeguarding global food security.

4. Conclusion

This study highlights the possible challenge of AF contamination in maize and other agricultural products, emphasizing the necessity for regular monitoring to ensure food safety. The investigation of weekly AF production over 7 weeks by the isolated toxigenic *A. flavus* strain from maize provides valuable insights into the formation of AFs. The validated approach for concurrent quantification and monitoring of AF production by *A. flavus* VKMN22 contaminated maize inoculated on agar was effective, simple, and rapid. The analytical method used complied with the analytical method performance guidelines set by European Commission. Successful quantification and monitoring of AFB_1 and AFB_2 concentration levels in maize grains inoculated with *A. flavus* on PDA media over seven weeks was achieved using this validated LC-MS method. AF production by *A. flavus* started from the first week, then increased linearly up to week five then reduced at weeks six and further reduced to 2005 μg/kg in week 7. Therefore, it is clear that biomonitoring of AF contamination in food samples provides essential data for comprehensive risk assessment, regulatory compliance, public health interventions, environmental monitoring, and quality control in agriculture.

CRediT authorship contribution statement

Viola O. Okechukwu: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Abidemi P. Kappo:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Investigation, Data curation, Conceptualization. **Patrick B. Njobeh:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Messai A. Mamo:** .

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2024.100197.

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