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Optimization of DNA extraction and application of qPCR and ddPCR assays for detection of toxigenic *Aspergillus flavus* in hazelnut kernels

Alessia CASU[1], Giorgio CHIUSA[1], Paola BATTILANI[1,*], Hillary L. MEHL[2]

[1] *Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, Piacenza, 29122, Italy*
[2] *United States Department of Agriculture, Agricultural Research Service, Tucson, AZ, United States of America*
*Corresponding author. E-mail: paola.battilani@unicatt.it

Summary. Aflatoxin contamination in hazelnuts, primarily caused by *Aspergillus flavus*, poses significant risks to food safety and public health, requiring highly sensitive and robust toxin detection strategies. While conventional culturing techniques remain relevant, they are time-consuming and prone to misidentifications. A molecular workflow for early detection of aflatoxigenic *A. flavus* in hazelnuts was developed and validated, combining an optimized DNA extraction protocol with quantitative PCR (qPCR) and droplet digital PCR (ddPCR) assays. Four DNA extraction methods were compared for their DNA yields and purity. DNA extraction protocol was optimized introducing a Tween-80 separation step, and was tested on hazelnuts artificially inoculated with aflatoxigenic *A. flavus* conidia. The optimized protocol was then validated for naturally contaminated hazelnut samples to assess its practical applicability, and to benchmark the performance of qPCR and ddPCR on real samples. The optimized protocol gave yield, purity and amplifiability, and appeared more appropriate for detecting aflatoxigenic *A. flavus* DNA in complex food matrices such as hazelnuts. The qPCR and ddPCR protocols detected target DNA, with ddPCR offering enhanced sensitivity and superior analytical performance. The developed protocol showed an increased sensitivity and quantification precision compared with previously developed methods. This research provides a validated molecular workflow for the early and sensitive detection of *A. flavus* in hazelnuts, offering a tool for preventive food safety monitoring and supporting aflatoxin risk assessment strategies for the hazelnut value chain.

Keywords. Aflatoxins, food safety, molecular diagnostics, digital PCR, fungal contamination.

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ORCID:
AC: 0009-0000-5044-4329
PB: 0000-0003-1287-1711
HLM: 0000-0001-8570-3562

INTRODUCTION

Hazelnut (*Corylus avellana L.*) is an economically important tree nut, widely used in chocolate and confectionery industries. It has high nutritional

and lipidic content, but it is susceptible to fungal contamination during growth, harvest, processing and storage (Kabak, 2016; Şen *et al.,* 2025). *Aspergillus flavus* is a significant threat to the safety of human and animal food due to its capacity to synthetize aflatoxins (AFs), which are toxic and carcinogenic secondary metabolites. Among these, aflatoxin B_1 (AFB_1) is recognized as a Group 1 human carcinogen by the International Agency for Research on Cancer (IARC, 1993; Rushing *et al*., 2019).

The presence of AFs poses serious health concerns and has major economic implications. The European Union has set strict limits (5 μg kg^{-1} for AFB_1; 10 μg kg^{-1} for total AFs, in nuts for direct consumption; European Commission (EC), 2023), and products exceeding these thresholds are routinely rejected, leading to trade losses. Notifications from the Rapid Alert System for Food and Feed (RASFF) indicate recurrent AFs contamination in hazelnuts, particularly from Turkey, Georgia, and Azerbaijan (RASFF, 2025). With climate change extending AFs risk into temperate areas, the challenge of ensuring hazelnut safety is increasing (Şen *et al.,* 2025).

Identifying *Aspergillus* species in food has relied on plate counts and culturing methods that focus on macro- and micro-morphological characteristics. These techniques are time-consuming and require skilled personnel for fungus identifications. Molecular methods mitigate the chances of incorrect identification, and efforts have been made to design techniques that are highly specific and sensitive for detecting and quantifying aflatoxigenic *Aspergillus* species. Several methods have been developed for detection of these fungi, using polymerase chain reaction (PCR) and quantitative PCR (qPCR) assays (Shapira *et al.,* 1996; Chen *et al.,* 2002; Scherm *et al.,* 2005; Latha *et al*., 2008; Degola *et al.,* 2009; Luo *et al*., 2009; Passone *et al.,* 2010; Sardiñas *et al.,* 2011; Rodríguez *et al*., 2012; Shweta *et al.,* 2013; Ahmad *et al*., 2014; Mahmoud, 2015; Mitema *et al.,* 2019; Ortega *et al.,* 2020; Garcia-Lopez *et al.,* 2021; Leharanger *et al.,* 2024), or loop-mediated isothermal amplification (LAMP) assays (Luo *et al.,* 2012; Liu *et al.,* 2014; Luo *et al.,* 2014; Niessen *et al*., 2018; Douksouna *et al.,* 2020; Ortega *et al*., 2020). However, applications for hazelnuts remain limited (Gallo *et al.,* 2010; Hamed *et al*., 2016; Ortega *et al.,* 2020; Habibi, 2021; Lombardi *et al.,* 2022; Nooralden *et al*., 2022; Hassan *et al*., 2023; Aghayev *et al.,* 2025).

Droplet digital PCR (ddPCR) has gained prominence as a sensitive and accurate method for the detection and quantification of microbial populations, and some assays for *A. flavus* are already available (Hua *et al.,* 2018; Schamann *et al.,* 2022; Wang *et al.,* 2022; Palumbo *et al.,* 2023). Unlike qPCR, ddPCR enables absolute quantification of target DNA, by partitioning PCR reactions into thousands of nanoliter-sized droplets. In addition, ddPCR offers high sensitivity, making it suitable for identifying low concentrations of fungal DNA, which are often encountered in the initial phases of contamination or within food matrices with minimal fungal presence. Resilience of ddPCR where PCR inhibitors (polyphenols, fats, polysaccharides) are present, which commonly occurs in complex food matrices such as hazelnuts, further enhances ddPCR effectiveness.

The objectives of the present study were: to (i) compare different DNA extraction protocols for hazelnut matrices and evaluate their DNA purity and yields; (ii) assess the sensitivity and reliability of qPCR and ddPCR assays for *A. flavus* detection, including determination of their analytical detection limits of detection; (iii) optimize the extraction procedure with additional pretreatment steps to reduce inhibition and improve *A. flavus* recovery; and (iv) validate the optimized workflow for naturally *A. flavus* contaminated hazelnut samples, benchmarking molecular assays against conventional measures.

MATERIALS AND METHODS

Fungus strains and conidium suspension preparation

Aflatoxigenic *A. flavus* isolate AF13, from the USDA-ARS Aflatoxin Reduction in Crops Laboratory (Tucson, Arizona), was used for artificial inoculations of hazelnut samples (Cotty *et al*., 1993). DNA from the non-aflatoxigenic isolate AF36 of *A. flavus*, and a strain of *Fusarium verticillioides* from the same laboratory collection, were included as negative controls in qPCR and ddPCR assays.

To produce conidium suspensions, isolate AF13 was cultured on 5/2 agar supplemented with salt (5% V8 vegetable juice, 2% agar, 2% NaCl, pH 5.2). After 7 d incubation at 31°C, conidia were harvested using sterile cotton swabs, and were suspended in 10 mL water solution containing 0.01% Tween-80. Subsequently, 1 mL of this suspension was transferred to a tube containing 5 mL of sterilized water and 6 mL of pure ethanol (Sigma-Aldrich). Turbidity of the conidium suspension was measured using a TB300IR turbidimeter (Tintometer GmbH), and conidium concentration was calculated based on a nephelometric turbidity unit (NTU) and a colony forming units (CFUs) per mL standard curve, where CFU mL^{-1} = NTU (5.0×10^4) (Mehl *et al.,* 2010). The conidium suspension was diluted with sterile water to final concentrations of 1.2×10^6, 1.2×10^5, or 1.2×10^4 conidia mL^{-1}. These suspensions were used to artificially infest non-contaminated hazelnut samples in subsequent experiments.

Hazelnut samples

Two categories of hazelnut kernels were used in this study. Commercially available raw hazelnuts, purchased from the U.S. market, were supplied as pre-packed, deshelled kernels. These were ground into flour and considered as non-contaminated material, and the flour was subsequently used for artificial inoculations with AF13 conidia to optimize DNA extraction protocols and evaluate the limits of detection (LOD) of molecular assays.

In parallel, a total of ten hazelnut samples originating from local commercial markets in Azerbaijan, were collected from the 2023/2024 cropping season from the Khachmaz and Zaqatala regions. The samples, delivered in-shell after approx. 6 months of storage at room temperature (18–22°C), were each manually deshelled, ground into flour, and analyzed as naturally contaminated material with unknown levels of *A. flavus*. These samples were included to evaluate the initial DNA extraction performance and to validate the final optimized protocol.

All hazelnuts were ground into fine flour using the Grindomix GM200 knife mill (Retsch GmbH). Samples from Azerbaijan were analyzed by culture-based and molecular methods.

Culture-based assessments of fungal contamination

To estimate the initial fungal load in naturally contaminated hazelnuts, 1 g of hazelnut flour from each Azerbaijani sample was suspended in a final volume of 10 mL of sterile water, and was then homogenized using a Vortex ZX3 (Genelab Srl). Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) were prepared, and 500 µL from each dilution was plated onto each of three Petri plates containing Dichloran Rose Bengal Chloramphenicol (DRBC) agar (DRBC Agar Base; Biolife Italiana S.r.l), supplemented with 0.05 g L^{-1} chloramphenicol. The plates were then incubated at 31°C in the dark for 4 d, and *A. flavus* colonies were enumerated based on morphology. LOD was calculated on the most concentrated aliquot plated. One gram of sample was diluted 1:10 (0.1 g mL^{-1}), and 0.5 mL of this suspension was plated onto each of five replicate plates (volume of 2.5 mL, corresponding to 0.25 g of each sample). Assuming 1 CFU as the minimum detectable number, each LOD was calculated as 1 CFU per 0.25 g = 4 CFU g^{-1}.

DNA extraction tests

Four different DNA extraction methods were evaluated for their performance on naturally contaminated hazelnut samples. The methods were: the DNeasy Plant Pro Kit (Qiagen), the FastDNA Spin Kit (MP Biomedicals), the protocol described by Callicott *et al.* (2015), and a method based on InstaGene Matrix (Bio-Rad Laboratories).

The four DNA extraction methods were selected based on their documented use in mycological and food-related applications, particularly those involving *A. flavus*, and on their diversity of chemical–physical extraction principles. The DNeasy Plant Pro Kit was chosen because it is widely employed for genomic DNA extraction from filamentous fungi, and has demonstrated high performance and consistency across multiple fungal taxa (Conlon *et al.,* 2022). The DNeasy Plant Pro system has also been successfully applied for *A. flavus* detection in pure cultures, contaminated plant tissues, and food matrices (González-Salgado *et al.,* 2011). The FastDNA Spin Kit was included due to its extensive use in *A. flavus* research, showing high lysis efficiency and tolerance to inhibitory matrices. It has been successfully used to extract DNA from mycelia, conidia, soil, and nut-based matrices, such as pistachio kernels and hulls (Luo *et al.,* 2009; Mehl *et al.,* 2010; Grubisha *et al.,* 2015; Garcia-Lopez *et al.,* 2021). The Callicott *et al*. (2015) protocol was incorporated as a reference method, as it was specifically developed for *A. flavus* and is routinely employed in aflatoxin ecology and toxigenicity studies. The InstaGene Matrix (Bio-Rad) was included as a rapid, resin-based extraction method that is commonly used for preparing PCR-ready DNA from filamentous fungi, including *Aspergillus spp*., in clinical and food-related contexts (Ciardo *et al.,* 2007; Soliman *et al.,* 2015).

For the ten hazelnut samples from Azerbaijan, and for each of the four extraction methods, a subsample of hazelnut flour was taken, giving ten biological replicates analyzed using each of the four methods.

For the DNeasy Plant Pro Kit, 0.2 g of hazelnut flour was homogenized in 500 µL of lysis buffer (Solution CD1), using a TissueLyser II (Qiagen) set at 24 Hz for 2 × 2 min cycles. After centrifugation (12,000 × g for 2 min), the supernatant was purified through binding and washing steps following the manufacturer's guidelines. DNA was then eluted in 75 µL of Buffer EB, and was stored at -20°C until further analysis.

For the FastDNA Spin Kit, 0.2 g of hazelnut flour were added to Lysing Matrix A tubes with lysis and precipitation solutions (800 µL CLS-VF and 200 µL PPS), and was homogenized using a TissueLyser II (24 Hz, 2 × 2 min). After centrifugation to pellet debris, the supernatant was mixed with Binding Matrix and applied to SPIN Filters for sequential binding and washing steps.

DNA was eluted in 100 µL of DES after incubation at 55°C, and was stored at -20°C until further use.

For the Callicott *et al.* (2015) DNA extraction protocol, 0.2 g of each sample was combined with 450 µL of lysis buffer (30 mM Tris, 10 mM EDTA, 1% SDS, pH 8·0). The samples were then incubated in Thermomixer 5436 (Eppendorf Inc.) for 1 h at 60°C and 800 rpm. Following the removal of plant fragments using centrifugation, the supernatant was transferred to a new tube and DNA was precipitated with 4M ammonium acetate and 100% cold ethanol. The pellet was then air dried, and DNA was resuspended in 25 µL of sterile water.

For the InstaGene DNA extraction protocol, 0.10 mg of hazelnut flour was mixed with 40 µL of InstaGene Matrix (Bio-Rad Laboratories) in 0.2 mL capacity PCR strip tubes. DNA was then extracted by incubating the samples at 95°C for 10 min in an All In One Cycler PCR Thermocycler (Bioneer), followed by centrifugation at 3800 rpm for 3 min to remove plant particles. The supernatant was then transferred to a new strip tube with a final maximum volume of 20 µL (based on extraction efficiency).

DNA concentrations were measured with a Qubit 4 fluorometer, using the Qubit 1X dsDNA Broad Range (BR) Assay kit (Thermo Fisher Scientific). Quality of the DNA was assessed with a NanoDrop One Spectrophotometer (Thermo Fisher Scientific).

All values obtained from Qubit and Nanodrop measurements (expressed in ng DNA μL^{-1}) were multiplied by the total volume (µL) of each extract to obtain the total ng of DNA per sample.

qPCR and ddPCR assays

The qPCR assay was carried out using primers Fw-nomutB (5′-CTTGGTCTACCATTGTTTGG-3′) and RV-nomut267 (5′-GGTAGGCGTCGTGTCTAG-3′), targeting the aflC gene, which encodes a key enzyme in the aflatoxin biosynthesis pathway. This primer pair specifically amplifies a 284-bp fragment in *A. flavus* isolates that lack the AF36 SNP, allowing selective detection of aflatoxigenic strains (Garcia-Lopez *et al.,* 2021). PCR amplifications were each carried out with two technical replicates per sample in a CFX96 Touch Instrument (Bio-Rad). Reactions (each 20 µL) included 10 µL iTaq SYBR Green Supermix (Bio-Rad), 0.6 µL of each 10 µM primer, 2 µL of undiluted DNA template, and 6.8 µL of sterile water. To evaluate possible PCR inhibition, each sample was also tested in an inoculated version, where 2 µL of water was replaced with 2 µL of AF13 DNA (0.1 ng μL^{-1}, 0.2 ng per reaction, for a total of 5×10^3 copies per reaction). Cycling was proceeded using the following conditions: 95°C for 5 min, 40 cycles each of 95°C for 5 s and 64°C for 30 s, followed by melting curve analysis. Standard curves were constructed using serial dilutions of AF13 DNA (5.0×10^4 to 0.5 copies per reaction) performed in triplicate.

The ddPCR assay followed the outline by Schamann *et al.* (2022), using a Bio-Rad custom assay (ID: dMDS741862930), targeting a single nucleotide polymorphism (SNP) in the *pksA* gene that distinguishes between the functional gene in aflatoxigenic *A. flavus* (labelled with a HEX probe) versus the mutation that confers non-aflatoxigenicity in the *A. flavus* biocontrol strain AF36 (labelled with a FAM probe). Since the goal of the present study was detection of aflatoxigenic *A. flavus*, only HEX fluorescence was considered for quantifications. Each reaction mix contained 10 µL 2× ddPCR Supermix (no dUTP), 1 µL primer/probe mix, 1 µL enzyme mix (0.5 µL HaeIII, 0.1 µL Cutsmart buffer, 0.4 µL water), 6 µL water, and 2 µL of template DNA, for a total volume of 20 µL. Droplets were generated and amplified (95°C for 10 min; 40 cycles each of 94°C for 30 s, 53°C for 60 s; 98°C for 10 min), then read on a QX200 Droplet Reader. Data were analyzed using QuantaSoft software with default amplitude thresholds and manual confirmation of positive droplet separation.

Each qPCR and ddPCR assay included non-template controls (NTCs) and *F. verticillioides* DNA as negative controls. Additionally, ddPCR included AF13 and AF36 DNA as positive controls. All control DNAs were originally at 1 ng μL^{-1} and were each diluted 1:10 in sterile water to a working concentration of 0.1 ng μL^{-1} prior to use.

All raw data obtained from qPCR and ddPCR were converted to copies per g. For qPCR results, copies of DNA/µL = [Avogadro's constant × DNA conc. (ng μL^{-1})]/[genome size (bp) × 10^9 ng/g × average weight of bp]. Copies per µL were multiplied by µL of extract to obtain total copies, and this was then divided per g of extract to obtain copies g^{-1}. For ddPCR, copies per reaction value were multiplied by the reaction volume to obtain copies per reaction. This was then divided by µL of DNA extract per reaction to get copies per reaction extract. These were then multiplied by total volume (µL) of extract to obtain total copies, that were divided by the hazelnut quantity used for extraction to obtain copies per g of hazelnut.

Optimization of DNA extraction

Initial quantification results indicated that concentrations of *A. flavus* in DNA extracts were below detectable levels. To determine the limit of *A. flavus* propagule

quantification and potentially increase the concentration of *A. flavus* in DNA extracts, non-infested hazelnuts were artificially inoculated with AF13 conidium suspensions at initial concentrations of approx. 1.2×10^6, 1.2×10^5, or 1.2×10^4 conidia mL^{-1}. For inoculation, 200 µL of each suspension were thoroughly mixed with 2 g of ground hazelnut, resulting in final concentrations of approx. 1.2×10^5, 1.2×10^4, and 1.2×10^3 conidia g^{-1}. A control sample to which 200 µL of sterile water was added. DNA extractions were carried out immediately after inoculations. The 2 g of inoculated hazelnut flour were then added to 20 mL of 0.01% Tween-80 and mixed for 20 min at 175 rpm on a horizontal shaker (IKA Werke GmbH & Co.). Following mixing, the solid (kernel) and liquid (Tween-80 suspension) fractions were manually separated by carefully pouring the liquid fraction into a new 50 mL tube while leaving the fatty deposit in the original tube. The liquid fraction was first centrifuged at 7,000 × g for 10 min, and the resulting pellet was transferred into 2 mL a microcentrifuge tube. In parallel, the kernel fraction was directly transferred into a 2 mL tube without prior centrifugation. Both fractions were then subjected to a second centrifugation at 8,000 × g for 10 min. After this step, resulting pellets from the liquid and the kernel fractions (approx. 500 to 600 µL) were collected and used for DNA extraction.

DNA was extracted using DNeasy Plant Pro and FastDNA Spin kits, with modifications: tough microorganism lysing tubes (Revvity) substituted standard disruption tubes, and each tube was loaded with two 6 mm ceramic beads (MP Biomedicals) to improve cell disruption. For the DNeasy protocol, the CD1 buffer volume was increased to 600 µL. Mechanical lysis was carried out using a Tissuelyser (Qiagen) at 25 Hz for 2 min. The experiment was carried out in triplicate, and DNA quantities and quality were assessed following the procedures outlined above. Performance of the two DNA extraction protocols was evaluated using qPCR and ddPCR with the methods outlined above.

Validation of optimized protocol on naturally contaminated hazelnut samples

The DNeasy Plant Pro Kit optimized DNA extraction protocol (with modifications) was applied to the ten Azerbaijani hazelnut samples. From each sample, 2 g of hazelnut flour were suspended in 20 mL of 0.01% Tween-80, was then homogenized, and the liquid fraction was retained. Following sequential centrifugation (7,000 rcf for 10 min; 8,000 rcf for 10 min), approx. 500 to 600 µL of fungal pellet were collected and subjected to DNA extraction. The experiment was conducted in duplicate, and qPCR and ddPCR were both employed for DNA detections and quantification (as described above).

Statistical analyses

An initial statistical assessment compared the four DNA extraction methods, using quantitative and qualitative data from Nanodrop and Qubit measurements. These results underwent a univariate analysis of variance (UNIANOVA). Tukey's test was used to evaluate means and detect statistically significant differences. A subsequent analysis was conducted following the optimization of DNA extraction, incorporating quantitative and qualitative data from Nanodrop and Qubit alongside qPCR and ddPCR results. Considered factors included the extraction method (DNeasy or FastDNA), the spore inoculum concentration (1.2×10^5, 1.2×10^4, or 1.2×10^3 conidia g^{-1}), and the hazelnut fraction used for DNA extraction (liquid or kernel). UNIANOVA and Tukey's test were applied to assess means and reveal statistically significant differences.

The relationship between molecular detection results (qPCR and ddPCR copy numbers) and culture-based contamination levels (expected copies g^{-1} derived from CFU counts) was evaluated using correlation analyses. Pearson's rank correlation coefficient was calculated to assess linear associations amongst the data, and Spearman's rank correlation coefficient to assess monotonic associations. Prior to these analyses, data were $\log_{10}$-transformed to normalize distributions and stabilize variances. Correlations were computed using IBM SPSS Statistics software, version 29.0.1.0 (IBM Corp.), and statistical significance was determined at $P < 0.05$.

RESULTS

Fungal contamination in naturally infected hazelnut samples

Colonies were identified as *A. flavus* based on macroscopic and microscopic morphological characteristics, including colony colour and texture on DRBC agar, presence of radiate conidial heads, rough conidiophores, and globose vesicles, following standard taxonomic keys (Pitt *et al.,* 2009).

Fungus contamination among the ten naturally contaminated hazelnut samples ranged from non-detectable levels to a maximum of 1.5×10^3 CFU g^{-1}, with a mean value of approx. 3.9×10^2 CFU g^{-1}. In some cases, no colony growth was observed, indicating the presence of non-viable propagules, or absent fungal colonization (Table 1).

Table 1. Number of *Aspergillus flavus* colony-forming units per gram (CFU g-1) in hazelnut flour from ten naturally contaminated nut samples.

Sample	*A. flavus* (CFUs g^{-1})
1	3.17×10^2
2	1.24×10^2
3	< LOD[b]
4	5.20×10^2
5	5.00×10^1
6	2.00×10^1
7	< LOD
8	< LOD
9	1.53×10^3
10	< LOD

[a] CFUs were determined by serial dilution and plating on DRBC agar. Values represent the average CFU g^{-1} obtained from two replicate plates and three dilutions (10^{-1}, 10^{-2} and 10^{-3}).
[b] Limit of detection = 4 CFU g^{-1}.

Quantities and quality of DNA extracted from naturally infested hazelnut samples

To identify the most suitable DNA extraction protocol for detecting *A. flavus* in naturally contaminated hazelnut samples, four methods were evaluated, focusing on their DNA yield, purity, and compatibility with downstream molecular assays. While all protocols yielded measurable DNA from the samples, the protocols differed for consistency, purity, and PCR inhibition (Table 2; Supplementary Table S1).

Overall, total DNA yields measured by Nanodrop varied considerably among the extraction methods. The InstaGene Matrix protocol produced greater DNA quantities than the other three methods ($P < 0.01$), whereas the Callicott *et al.* (2015), FastDNA, and DNeasy kits yielded similar total DNA amounts to each other.

However, these elevated Nanodrop results were not consistent with Qubit quantification. Qubit measurements gave increased DNA recovery for the FastDNA and DNeasy kits compared with the Callicott and Cotty (2015) method (Table 2). No Qubit data were obtained for the InstaGene Matrix protocol, due to lack of sufficient DNA material.

For DNA purity, the A260/A280 ratios showed differences among the extraction methods ($P < 0.01$; Table 2). The DNeasy Plant Pro Kit gave the greatest purity values, while the InstaGene Matrix gave the least, indicating substantial protein contamination in the assessed samples. The A260/A230 ratios also differed ($P < 0.01$) across methods. The DNeasy Plant Pro Kit gave the greatest values, whereas the FastDNA Spin Kit gave low values. However, all four methods produced values below the optimal A260/ A230 level, indicating persistent carryover of organic residues or salts.

Analytical validation of qPCR and ddPCR assays

In qPCR, amplification was observed across five of the concentrations in the six-point ten-fold dilution series of AF13 DNA, from 5×10^4 copies down to 0.5 copies per reaction. The assay reliably detected DNA down to 50 copies per reaction, with amplification observed in all experimental replicates. At five copies per reaction, amplification became inconsistent, and no amplification was observed at 0.5 copies, establishing, under the tested condition, 50 copies per reaction as the limit of quantification (LOQ), and five copies per reaction as the limit of detection (LOD).

Non-template controls gave no amplifications, confirming absence of contamination or background signals. For *F. verticillioides*, two replicates showed no amplification, while one gave a non-specific melting peak, indicating that the assay discriminated positive signals from background or non-target amplifications.

ddPCR confirmed the specificity of probe-based detection. AF13 DNA generated a clear signal in channel 2 (HEX, targeting the aflatoxigenic strain), while AF36

Table 2. Mean DNA purity parameters (± standard deviations) from four assessed extraction methods. tested.

Method	Nanodrop[a] (total ng)	A260/A280	A260/A230	Qubit (total ng)
	**	**	**	**
Callicott *et al.* (2015)	$7.5 \times 10^3 \pm 1.9 \times 10^3$ b	1.53 ± 0.10 b	0.44 ± 0.05 b	$6.8 \times 10^2 \pm 3.5 \times 10^2$ b
DNeasy Plant Pro Kit (Qiagen)	$7.0 \times 10^2 \pm 2.0 \times 10^2$ b	2.07 ± 0.36 a	0.75 ± 0.39 a	$10.0 \times 10^2 \pm 2.9 \times 10^2$ b
FastDNA Spin Kit (MP Biomedicals)	$3.6 \times 10^3 \pm 4.6 \times 10^2$ b	1.65 ± 0.12 b	0.03 ± 0.01 c	$1.8 \times 10^3 \pm 2.8 \times 10^2$ a
InstaGene Matrix (Bio-Rad)	$5.0 \times 10^4 \pm 1.3 \times 10^4$ a	1.25 ± 0.12 c	0.31 ± 0.02 b	*n.a*

[a] Nanodrop and Qubit values are expressed as total ng per sample. n.a indicates data not measured/calculated.
** $P < 0.01$; Different letters accompanying means indicate statistically significant differences, according to Tukey's tests.

was detected exclusively in channel 1 (FAM, targeting the non-aflatoxigenic strain). No droplets were observed in either channel when using *F. verticillioides* DNA or sterile water.

Compatibility of extracted DNA with qPCR and ddPCR assays

The four DNA extraction methods were assessed for their compatibility with qPCR and ddPCR amplifications. For amplification efficiency, *A. flavus* DNA was detected by qPCR only where the fungus was added to samples, while naturally contaminated samples remained below the detection threshold for all four extraction methods. Among *A. flavus* inoculated samples, both the DNeasy and FastDNA kits yielded detectable amplification signals, while no amplification occurred with the Callicott *et al.* (2015) and InstaGene methods (Supplementary Table S1).

Quantitatively, DNeasy consistently yielded greater copy numbers per reaction, corresponding to approx. 40 to 60% of the expected added concentration, whereas FastDNA averaged 30 to 40% of the expected values. This difference reflects a lower degree of PCR inhibition in the DNeasy extracts than for the FastDNA extracts, likely due to increased DNA purity from the FastDNA system.

In contrast, ddPCR assays yielded no detectable *A. flavus* DNA in any of the naturally contaminated hazelnut samples. Inoculated controls were not included for ddPCR, as the method is largely unaffected by PCR inhibitors. Therefore, absences of amplification were probably true negatives rather than inhibition artifacts.

Optimization of DNA extraction using artificially infested hazelnuts

To refine the DNA extraction process and improve assay performance, hazelnuts were artificially infested with 1.2×10^5, 1.2×10^4, or 1.2×10^3 conidia g^{-1} of *A. flavus* AF13. Each sample was pre-treated with a Tween-80 solution to facilitate fungus recovery. After mixing on the horizontal shaker, the mixture divided into two fractions, one that was whitish liquid and the other a brown sedimented kernel fraction. The two fractions were manually separated by carefully pouring the liquid into a new 50 mL capacity tube, while leaving the plant residues in the original tube. The liquid fraction after being re-centrifuged, gave a whitish sediment corresponding to fat residues from the hazelnuts. The solid kernel fraction consisted of plant residues occupying only the tip of the tube. Both pellets (white sediment from liquid fraction, brown plant residue sediment from kernel fraction) were taken (~500 to 600 µL) and were analyzed independently (Supplementary Table S2).

According to UNIANOVA (Table 3, Supplementary Table S3), both the extraction method and the sample fraction data affected DNA yield and quality parameters, whereas inoculum concentration influenced only the quantification results obtained by qPCR and ddPCR.

DNA yield estimated by Nanodrop was similar ($P > 0.05$) among the methods. In contrast, Qubit measurements and purity ratios (A260/A280 and A260/A230) revealed distinctions between the extraction methods. The DNeasy kit produced DNA of greatest purity, with A260/A280 and A260/A230 ratios close to optimum values, whereas the FastDNA kit gave consistently lower purity values, indicating presence of protein and salt contaminants.

Fraction type also gave a statistically significant effect. Samples derived from the liquid fractions consistently provided greater DNA quantities and better amplification results than those obtained from the kernel fractions, confirming that the Tween-80 washing step improved recovery of fungal material. The method × fraction interaction was significant ($P < 0.05$) for some parameters, indicating that while both kits performed comparably in terms of yield, the DNeasy method was more effective in extracting clean, amplifiable DNA from the liquid fraction, and the FastDNA method tended to produce lower purity extracts, particularly from the kernel fraction (Figure 1).

Table 3. Summary of significant main effects identified by UNIANOVA across DNA yield, purity parameters, and molecular detection assays.

Factors	Nanodrop	A260/A280	A260/A230	Qubit	qPCR	ddPCR
Method (M)	*n.s*	*n.s*	$P < 0.01$	$P < 0.05$	$P < 0.01$	*n.s*
Concentration (C)	*n.s*	*n.s*	*n.s*	*n.s*	$P < 0.01$	$P < 0.01$
Fraction (F)	*n.s*	$P < 0.05$	*n.s*	$P < 0.01$	$P < 0.01$	$P < 0.05$

n.s. = non-significant ($P > 0.05$); * $P < 0.05$; ** $P < 0.01$.
Full numerical results, factor levels, and interaction terms (M × C, M × F, C × F, M × C × F) are reported in Supplementary Table S3.

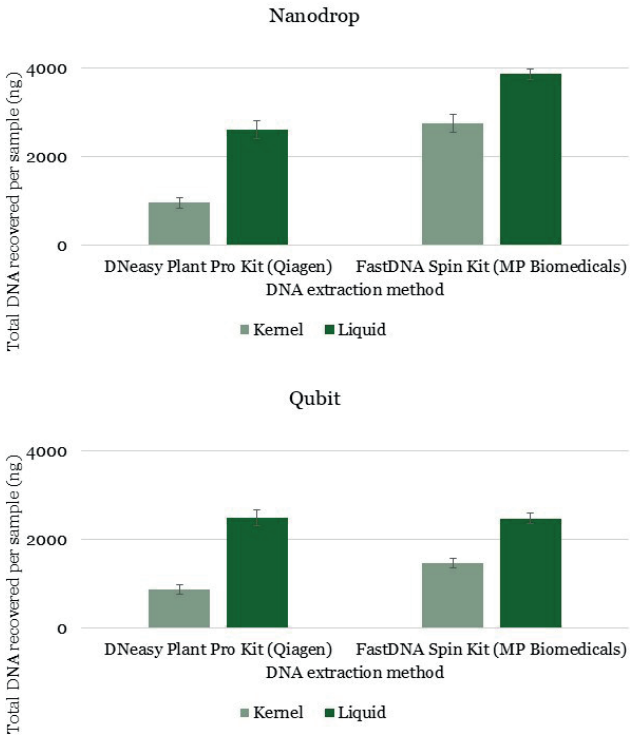

Figure 1. Mean total amounts of DNA per sample from Nanodrop and Qubit quantification for two DNA extraction methods (DNeasy Plant Pro Kit, FastDNA Spin Kit), for two hazelnut fractions (kernel or liquid). Bars indicate standard errors of means.

The qPCR and ddPCR data further confirmed these trends. Both assays detected *A. flavus* DNA in all conidium concentrations, but signal strength and consistency declined with decreasing inoculum. qPCR amplification was generally more robust than ddPCR for DNA extracted with the DNeasy kit and from the liquid fractions, reflecting greater recovery efficiency and lower inhibition. ddPCR results showed a similar pattern, detecting target DNA even at the lowest contamination level, confirming greater analytical sensitivity of ddPCR than for qPCR.

When comparing the measured and expected values (Supplementary Table S2), both qPCR and ddPCR results showed recovery ratios below 1 across all treatments, indicating that only a small fraction of the expected target DNA was recovered after extraction. Nevertheless, the DNeasy kit consistently produced greater measured/expected ratios than the FastDNA kit, particularly for the liquid fraction, suggesting a more effective recovery of amplifiable DNA and reduced inhibition for the DNeasy than the FastDNA kit.

The FastDNA kit, in contrast, yielded lower and more variable ratios than the DNeasy kit. Although total DNA quantities measured fluorometrically were comparable between the two kits, these values may reflect plant-derived DNA. The lower measured/expected ratios obtained with FastDNA therefore indicate less efficient extraction of *A. flavus* DNA rather than reduced availability of amplifiable templates.

For both extraction methods, the absolute amount of target DNA detected decreased with decreasing conidium concentration, as was expected. However, recovery efficiency (measured/expected ratios) remained stable across concentrations. Notably, the DNeasy kit maintained detectable amplification even at the lowest inoculum level, whereas FastDNA frequently approached the detection limit.

Validation of the optimized protocol on naturally contaminated hazelnut samples

To validate the optimized extraction protocol, the ten naturally contaminated hazelnut samples were reanalyzed using the DNeasy Plant Pro Kit, following sample pre-treatment with Tween-80 and selective recovery of the liquid fractions. Overall, the results confirmed that the optimized protocol efficiently extracted amplifiable DNA from naturally contaminated material, yielding consistent quantities of satisfactory purity suitable for downstream molecular analyses (Supplementary Table S4). Both Nanodrop and Qubit measurements showed reproducible DNA recovery across samples, while A260/A280 ratios remained close to the ideal value for pure DNA, indicating minimal protein contamination. The A260/A230 ratios were more variable among samples, reflecting the heterogeneous composition and matrix complexity of naturally contaminated nuts.

qPCR detection confirmed the presence of *A. flavus* DNA in most samples, although copy numbers were generally low, consistent with the low fungal counts in naturally contaminated material (Table 4). Both qPCR and ddPCR results correlated positively with culture-based CFU-derived estimates. The correlation was stronger for qPCR (Spearman's $\rho = 0.77$, $P = 0.07$) than for ddPCR ($\rho = 0.66$, $P = 0.16$), indicating an overall positive relationship between molecular and culture-based detections, although these were not statistically significant due to the limited number of naturally contaminated samples. Some samples that yielded no detectable colonies on agar plates gave qPCR and ddPCR signals, suggesting that the molecular assays detected non-culturable or residual fungal DNA that remained undetected by culture-based methods.

ddPCR analyses supported these results, providing absolute quantification of *A. flavus* DNA with slightly

Table 4. Quantification and quality assessment of DNA extracted from ten naturally contaminated hazelnut samples using the optimized DNeasy Plant Pro Kit protocol.

Sample	qPCR (copies g^{-1})[a]	ddPCR (copies g^{-1})[a]	Expected copies per g[b]
1	$1.8 \times 10^2 \pm 1.6 \times 10^2$	$1.5 \times 10^2 \pm 1.1 \times 10^2$	3.2×10^2
2	$6.4 \times 10^1 \pm 1.3 \times 10^2$	$2.9 \times 10^1 \pm 5.8 \times 10^1$	1.2×10^2
3	$3.1 \times 10^2 \pm 2.7 \times 10^2$	$1.5 \times 10^2 \pm 8.0 \times 10^1$	*n.d*
4	$1.3 \times 10^2 \pm 2.5 \times 10^2$	$5.2 \times 10^1 \pm 3.8 \times 10^1$	5.2×10^2
5	$6.3 \times 10^1 \pm 8.8 \times 10^1$	$2.7 \times 10^1 \pm 3.3 \times 10^1$	5.0×10^1
6	$2.3 \times 10^2 \pm 1.3 \times 10^2$	$9.2 \times 10^1 \pm 5.2 \times 10^1$	2.0×10^1
7	$2.4 \times 10^1 \pm 4.8 \times 10^1$	$2.0 \times 10^1 \pm 2.3 \times 10^1$	*n.d*
8	$1.5 \times 10^2 \pm 3.0 \times 10^2$	$1.1 \times 10^1 \pm 2.3 \times 10^1$	*n.d*
9	$1.2 \times 10^3 \pm 1.8 \times 10^3$	$1.2 \times 10^2 \pm 1.3 \times 10^1$	1.5×10^3
10	*n.d*	$2.1 \times 10^1 \pm 4.1 \times 10^1$	*n.d*

[a] qPCR and ddPCR were carried out on each sample.
[b] Expected copies are expressed in CFU g^{-1}, and were derived from plate counting analysis.
All values are means ± standard deviations from two independent replicates. N.d indicates where *A. flavus* was not detected through plate counting or that its DNA was not measured using qPCR.

greater detection consistency compared to qPCR. ddPCR confirmed low but measurable levels of target DNA across most samples, even when qPCR amplification was weak or absent, highlighting the superior sensitivity of droplet-based quantification.

When comparing molecular quantification with culture-derived estimates (CFU g^{-1}), the measured/expected ratios were generally below 1.0, indicating that the amount of amplifiable DNA was less than predicted based on counts of viable conidia. In a few samples, ratios could not be calculated (*n.a.*), corresponding to cases with zero CFU counts but detectable DNA.

DISCUSSION

Presence of aflatoxigenic *A. flavus* in hazelnuts is a persistent challenge for food safety, particularly due to the complex nature of the nut matrix and the typically low levels of fungal contamination encountered in commercial products (Gallo *et al.,* 2010).

Results from the present study demonstrate that optimization of DNA extraction is important for overcoming matrix-related limitations for toxigen detection. By systematically comparing four extraction protocols and introducing a Tween-80 washing step to separate the liquid and kernel fractions, it was possible to improve DNA yield and purity. Liquid fractions, in particular, concentrated most of the fungus spores while minimizing co-extraction of inhibitory compounds such as lipids and polysaccharides. These results highlight how simple pre-processing adjustments can enhance nucleic acid recovery and PCR detection performance.

When compared to previous research, this study represents a significant methodological advancement. Most previous studies investigating *A. flavus* in hazelnuts have relied on artificially inoculated hazelnut kernels or pre-cultured fungal biomass, rather than on direct analysis of the nut matrices. Gallo *et al.* (2010) examined fungal metabolism using hazelnut-based culture media, while Ortega *et al.* (2020) tested qPCR assays mostly on artificially inoculated kernels. Similarly, Hamed *et al.* (2016) and Hassan *et al.* (2023) extracted DNA from cultured mycelia, and Lombardi *et al.* (2022) characterized fungal communities of ready-to-eat hazelnuts using a polyphasic, culture-dependent approach that combined morphological and molecular identification of isolated colonies. In contrast, the results from the present study indicate that fungus cultivation is not required, but that direct targeting *A. flavus* DNA within hazelnut matrices can provide sensitive (low contamination) detection of aflatoxigenic strains.

Among the evaluated protocols, the DNeasy Plant Pro Kit provided the best compromise between yield, purity, and PCR compatibility. Column-based methods minimized contamination from proteins and organic residues, as indicated by stable A260/A280 ratios and reduced PCR inhibition. This extraction protocol was also effective in artificially contaminated hazelnuts and naturally infected samples, demonstrating robustness across contamination levels and matrix conditions.

Both qPCR and ddPCR assays successfully detected *A. flavus* DNA in inoculated samples, though ddPCR had superior analytical sensitivity and quantification accuracy, particularly at low inoculum levels. Unlike qPCR, ddPCR does not rely on calibration curves, and is less affected by PCR inhibitors, allowing for absolute quantification even in inhibitor-rich matrices such as hazelnuts. These results align with previous results in rice- and wheat-derived food matrices (Wang *et al.,* 2022), where ddPCR consistently outperformed qPCR in detecting low contamination levels of toxigenic fungi. Nevertheless, qPCR remains a highly practical tool for large-scale screening, providing rapid and cost-effective results suitable for industrial quality control *(*Shang *et al*., 2025). Both techniques are complementary: qPCR for high-throughput surveillance and ddPCR for confirmatory trace-level quantification.

The validation of the optimized extraction method on naturally contaminated hazelnuts confirmed its applicability under real conditions. Both qPCR and ddPCR

detected *A. flavus* DNA in multiple samples, often correlating with CFU estimates. In several cases, molecular detection was achieved even when CFUs were not detected, indicating that the method can identify non-culturable or residual fungal DNA that remains undetectable by classical microbiological assays. The number of naturally contaminated samples (n = 10) was limited, as the primary aim of this study was methodological rather than epidemiological. Samples were selected to represent commercial material, originating from different production batches and showing variable contamination levels. The sample set was therefore considered sufficient to evaluate robustness, sensitivity, and practical applicability of optimized DNA extraction and molecular detection. Nevertheless, larger surveys than for the present study, covering additional origins, seasons, and storage conditions, will be necessary to further consolidate quantitative performance of PCR detection under diverse conditions.

An important limitation of DNA-based detection is that presence of aflatoxigenic *A. flavus* does not directly imply active AFs production or presence of AFs in analyzed samples. AFs biosynthesis depends on multiple environmental and physiological factors, and DNA detection alone cannot distinguish between toxigenic potential and actual toxin accumulation. Consequently, molecular detection should be used as an early warning and risk-indication tool that complements chemical aflatoxin analysis (Northolt *et al.,* 1977; Caceres *et al.,* 2020).

In conclusion, this study has provided a validated and matrix-adapted molecular workflow for sensitive detection of aflatoxigenic *A. flavus* in hazelnut kernels. By integrating optimized DNA extraction with complementary qPCR and ddPCR assays, the proposed approach offers a practical tool for preventive monitoring and risk assessment in hazelnut supply chains. Beyond its analytical performance, the workflow has clear operational relevance. Its sensitivity can enable early detection of aflatoxigenic *A. flavus* in incoming batches, supporting risk-based acceptance decisions and strengthening HACCP monitoring at critical control points. Future studies may extend this framework to other mycotoxigenic species and food matrices, strengthening the role of molecular diagnostics in integrated food safety management systems.

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AUTHOR CONTRIBUTIONS

A. Casu: Conceptualization (equal), Data Curation (lead), Formal Analysis (equal), Investigation (lead), Validation (lead), Visualization (lead), Writing – Original Draft Preparation (lead); G. Chiusa: Methodology (supporting), Writing – Review & Editing (equal); P. Battilani: Conceptualization (equal), Methodology (supporting), Project Administration (equal), Supervision (equal), Writing - Review & Editing (equal); H. L. Mehl: Conceptualization (equal), Formal Analysis (equal), Funding Acquisition (lead), Methodology (lead), Project Administration (equal), Resources (lead), Supervision (equal), Validation (supporting), Writing – Review & Editing (equal).

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LITERATURE CITED

Aghayev F., Bandyopadhyay R., Battilani P., Ortega-Beltran A., 2025. Mycotoxin contamination of hazelnut grown in Azerbaijan and *Aspergillus* communities associated with the crop. *Plant Disease* https://doi.org/10.1094/pdis-12-24-2623-SC

Ahmad M.M., Ahmad M., Ali A., Hamid R., Javed S., Abdin M.Z., 2014. Detection of *Aspergillus flavus* and *Aspergillus parasiticus* from aflatoxin-contaminated peanuts and their differentiation using PCR-RFLP. *Annals of Microbiology* 64(4): 1597–1605. https://doi.org/10.1007/S13213-014-0803-5

Caceres I., Khoury A. Al, El Khoury R., Lorber S., Bailly J.D., 2020. Aflatoxin biosynthesis and genetic regulation: a review. *Toxins* 12(3): 150. https://doi.org/10.3390/toxins12030150

Callicott K.A., Cotty P.J., 2015. Method for monitoring deletions in the aflatoxin biosynthesis gene cluster of *Aspergillus flavus* with multiplex PCR. *Letters in Applied Microbiology* 60(1): 60–65. https://doi.org/10.1111/lam.12337

Chen R.S., Tsay J.G., Huang Y.F., Chiou R.Y.Y., 2002. Polymerase chain reaction–mediated characterization of molds belonging to the *Aspergillus flavus* group and detection of *Aspergillus parasiticus* in peanut kernels by a multiplex polymerase chain reaction. *Journal of Food Protection* 65(5): 840–844. https://doi.org/10.4315/0362-028X-65.5.840

Ciardo D.E., Schär G., Altwegg M., Böttger E.C., Bosshard P.P., 2007. Identification of moulds in the diagnostic laboratory: an algorithm implementing molecular and phenotypic methods. *Diagnostic Microbiology and Infectious Disease* 59(1): 49–60. https://doi.org/10.1016/j.diagmicrobio.2007.04.020

Conlon B.H., Schmidt S., Poulsen M., Shik J.Z., 2022. Orthogonal protocols for DNA extraction from filamentous fungi. *STAR Protocols* 3(1): 101126. https://doi.org/10.1016/j.xpro.2022.101126

Cotty P.J., Bayman P., 1993. Competitive exclusion of a toxigenic strain of *Aspergillus flavus* by an atoxigenic strain. *Phytopathology* 83: 1283–1287. https://doi.org/10.1094/Phyto-83-1283

Degola F., Berni E., Spotti E., Ferrero I., Restivo F.M., 2009. Facing the problem of "false positives": reassessment and improvement of a multiplex RT-PCR procedure for the diagnosis of *A. flavus* mycotoxin producers. *International Journal of Food Microbiology* 129(3): 300–305. https://doi.org/10.1016/j.ijfoodmicro.2008.12.016

Douksouna Y., Kwallah A.O., Nyerere A., Runo S., Ambang Z., 2020. Application and evaluation of the loop-mediated isothermal amplification assay for the detection of aflatoxigenic fungi contaminants of rice grains in Kenya. *Plant Pathology & Microbiology* 11: 491.

European Commission (EC), 2023. Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006. *Official Journal of the European Union* L119: 103–127.

Gallo A., Epifani F., Bonsegna S., Pascale M., Santino A., Perrone G., 2010. Analysis of genes early expressed during *Aspergillus flavus* colonisation of hazelnut. *International Journal of Food Microbiology* 137(1): 111–115. https://doi.org/10.1016/j.ijfoodmicro.2009.11.010

Garcia-Lopez M.T., Luo Y., Ortega-Beltran A., Jaime R., Moral J., Michailides T.J., 2021. Quantification of the aflatoxin biocontrol strain *Aspergillus flavus* AF36 in soil and in nuts and leaves of pistachio by real-time PCR. *Plant Disease* 105(6): 1765–1772. https://doi.org/10.1094/pdis-05-20-1097-RE

González-Salgado A., González-Jaén T., Vázquez C., Patiño B., 2011. Highly sensitive PCR-based detection specific to *Aspergillus flavus*. *Methods in Molecular Biology* 739: 211–216. https://doi.org/10.1007/978-1-61779-102-4_19

Habibi A., 2021. *Aspergillus* species in retail samples of pistachio, walnut, and hazelnut in Kerman, Iran. *Mycologia Iranica* 8(2): 77–94. https://doi.org/10.22092/mi.2022.359369.1223

Hamed N.A.S., Murad A.F., Abdul-Rahim E.A.W., 2016. Molecular diagnosis of aflatoxigenic *Aspergillus flavus* isolated from nuts. *Research Journal of Environmental Toxicology* 10(1): 39–45.

Hassan K.I., Hama Sharef P., 2023. Identification of fungi associated with hazelnuts and determination of their mycotoxin. *IOP Conference Series: Earth and Environmental Science* 1213(1): 012074. https://doi.org/10.1088/1755-1315/1213/1/012074

Hua S.S.T., Palumbo J.D., Parfitt D.E., Sarreal S.B.L., O'Keeffe T.L., 2018. Development of a droplet digital PCR assay for population analysis of aflatoxigenic and atoxigenic *Aspergillus flavus* mixtures in soil. *Mycotoxin Research* 34(3): 187–194. https://doi.org/10.1007/S12550-018-0313-6

IARC, 1993. *Monographs on the Evaluation of Carcinogenic Risks to Humans*. Vol. 56. International Agency for Research on Cancer, Lyon, France.

Kabak B., 2016. Aflatoxins in hazelnuts and dried figs: occurrence and exposure assessment. *Food Chemistry* 211: 8–16. https://doi.org/10.1016/j.foodchem.2016.04.141

Latha R., Manonmani H.K., Rati E.R., 2008. Multiplex PCR assay for the detection of aflatoxigenic and non-aflatoxigenic *Aspergilli*. *Research Journal of Microbiology* 3(3): 136–142. https://doi.org/10.3923/jm.2008.136.142

Leharanger A., Paumier D., Orlando B., Bailly S., Valade R., 2024. Two new qPCR assays for detecting and quantifying the *Aspergillus flavus* and *Aspergillus parasiticus* clades in maize kernels. *Plant Pathology* 73(9): 2372–2381. https://doi.org/10.1111/ppa.13982

Liu P., Li B., Yin R., Weng Q., Chen Q., 2014. Development and evaluation of ITS- and aflP-based LAMP assays for rapid detection of *Aspergillus flavus* in food samples. *Canadian Journal of Microbiology* 60(9): 579–584. https://doi.org/10.1139/cjm-2014-0202

Lombardi S.J., Pannella G., Tremonte P., Mercurio I., Vergalito F., Coppola R., 2022. Fungi occurrence in ready-to-eat hazelnuts (*Corylus avellana*) from

different boreal hemisphere areas. *Frontiers in Microbiology* 13: 900876. https://doi.org/10.3389/fmicb.2022.900876

Luo Y., Gao W., Doster M., Michailides T.J., 2009. Quantification of conidial density of *Aspergillus flavus* and *A. parasiticus* in soil from almond orchards using real-time PCR. *Journal of Applied Microbiology* 106(5): 1649–1660. https://doi.org/10.1111/j.1365-2672.2008.04132.x

Luo J., Vogel R.F., Niessen L., 2012. Development and application of a loop-mediated isothermal amplification assay for rapid identification of aflatoxigenic molds and their detection in food samples. *International Journal of Food Microbiology* 159(3): 214–224. https://doi.org/10.1016/j.ijfoodmicro.2012.08.018

Luo J., Taniwaki M.H., Iamanaka B.T., Vogel R.F., Niessen L., 2014. Application of loop-mediated isothermal amplification assays for direct identification of pure cultures of *Aspergillus flavus*, *A. nomius* and *A. caelatus* and for their rapid detection in shelled Brazil nuts. *International Journal of Food Microbiology* 172: 5–12. https://doi.org/10.1016/j.ijfoodmicro.2013.12.001

Mahmoud M.A., 2015. Detection of *Aspergillus flavus* in stored peanuts using real-time PCR and expression of aflatoxin genes in toxigenic and atoxigenic isolates. *Foodborne Pathogens and Disease* 12(4): 289–296. https://doi.org/10.1089/fpd.2014.1854

Mitema A., Okoth S., Rafudeen S.M., 2019. The development of a qPCR assay to measure *Aspergillus flavus* biomass in maize and the use of a biocontrol strategy to limit aflatoxin production. *Toxins* 11(3): 179. https://doi.org/10.3390/toxins11030179

Niessen L., Bechtner J., Fodil S., Taniwaki M.H., Vogel R.F., 2018. LAMP-based group-specific detection of aflatoxin producers within *Aspergillus* section *Flavi* in food raw materials, spices and dried fruit using neutral red for visible-light signal detection. *International Journal of Food Microbiology* 266: 241–250. https://doi.org/10.1016/j.ijfoodmicro.2017.12.013

Northolt M.D., Van Egmond H.P., Paulsch W.E., 1977. Differences between *Aspergillus flavus* strains in growth and aflatoxin B1 production in relation to water activity and temperature. *Journal of Food Protection* 40(11): 778–781. https://doi.org/10.4315/0362-028X-40.11.778

Nooralden Z.N., Mohammed H.A., 2022. Isolation and identification of fungi associated with walnuts, almonds, hazelnuts, cashews, pistachios and peanuts using molecular diagnostics (PCR) in local markets of Kirkuk Governorate, Iraq. *Journal of Pharmaceutical Negative Results* 13: 1–8.

Ortega S.F., Siciliano I., Prencipe S., Gullino M.L., Spadaro D., 2020. Development of PCR, LAMP and qPCR assays for the detection of aflatoxigenic strains of *Aspergillus flavus* and *A. parasiticus* in hazelnut. *Toxins* 12(12): 757. https://doi.org/10.3390/toxins12120757

Passone M.A., Rosso L.C., Ciancio A., Etcheverry M., 2010. Detection and quantification of *Aspergillus* section *Flavi* spp. in stored peanuts by real-time PCR of the *nor-1* gene and effects of storage conditions on aflatoxin production. *International Journal of Food Microbiology* 138(3): 276–281. https://doi.org/10.1016/j.ijfoodmicro.2010.01.003

Pitt J.I., Hocking A.D., 1997. *Fungi and food spoilage*. Blackie Academic & Professional, London, UK. https://doi.org/10.1007/978-1-4615-6391-4

RASFF, 2025. Rapid Alert System for Food and Feed. Available at: https://webgate.ec.europa.eu/rasff-window/screen/search (accessed 27 October 2025).

Rodríguez A., Rodríguez M., Luque M.I., Martín A., Córdoba J.J., 2012. Real-time PCR assays for detection and quantification of aflatoxin-producing molds in foods. *Food Microbiology* 31(1): 89–99. https://doi.org/10.1016/j.fm.2012.02.009

Rushing B.R., Selim M.I., 2019. Aflatoxin B1: a review on metabolism, toxicity, occurrence in food, occupational exposure and detoxification methods. *Food and Chemical Toxicology* 124: 81–100. https://doi.org/10.1016/j.fct.2018.11.047

Sardiñas N., Vázquez C., Gil-Serna J., González-Jaén M.T., Patiño B., 2011. Specific detection and quantification of *Aspergillus flavus* and *Aspergillus parasiticus* in wheat flour by SYBR® Green quantitative PCR. *International Journal of Food Microbiology* 145(1): 121–125. https://doi.org/10.1016/j.ijfoodmicro.2010.11.041

Scherm B., Palomba M., Serra D., Marcello A., Migheli Q., 2005. Detection of transcripts of the aflatoxin genes *aflD*, *aflO* and *aflP* by reverse transcription–polymerase chain reaction allows differentiation of aflatoxin-producing and non-producing isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. *International Journal of Food Microbiology* 98(2): 201–210. https://doi.org/10.1016/j.ijfoodmicro.2004.06.004

Shapira R., Paster N., Eyal O., Menasherov M., Mett A., Salomon R., 1996. Detection of aflatoxigenic molds in grains by PCR. *Applied and Environmental Microbiology* 62(9): 3270–3273. https://doi.org/10.1128/AEM.62.9.3270-3273.1996

Shweta S., Madhavan S., Paranidharan V., Velazhahan R., 2013. Detection of *Aspergillus flavus* in maize kernels by conventional and real-time PCR assays. *International Food Research Journal* 20(6): 3329–3335.